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The Effect of Acute Ethanol Exposure on the Male Rodent Reproductive Axis

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THE EFFECT OF ACUTE ETHANOL EXPOSURE ON
THE MALE RODENT REPRODUCTIVE AXIS

by

Margaret Mary Halloran

A Dissertation submitted to
the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy
January 1994

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To Brian and Shane

ABSTRACT

THE EFFECT OF ACUTE ETHANOL EXPOSURE ON THE MALE RODENT REPRODUCTIVE AXIS

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are pituitary hormones necessary for the initiation and maintenance of reproductive processes. They are glycoprotein hormones synthesized in the gonadotrope cells of the anterior pituitary. One of the major regulators of LH and FSH synthesis and secretion is luteinizing hormone releasing hormone (LHRH).

The focus of this research was to study the effect of ethanol on these hormones: LH, FSH, and LHRH. An acute ethanol model was used, in which treatment was a single intraperitoneal injection of an ethanol solution. Controls were given an injection of saline. Sprague Dawley male rats were castrated two weeks before ethanol treatment to allow for an increase in the levels of LH and FSH.

The impact of ethanol (EtOH) on the male rodent reproductive axis had been characterized with a suppression of LH and FSH levels found in the serum. The mechanism of this suppression was extensively examined at the level of gene expression for LH and FSH. Acute EtOH studies of LH showed decreased expression of β -LH mRNA, no change in expression of α -LH mRNA, decreased serum LH, and increased pituitary content of LH. A similar decrease in FSH

levels in serum was seen. However, there was no change in intrapituitary FSH content or β -FSH mRNA levels. This data suggested that there was not a global effect of ethanol on the pituitary gonadotropin cells. Further analysis of β -LH expression after ethanol exposure, using polysome distribution analysis, has shown a decrease in translational efficiency. The impact of EtOH on the hypothalamus was not significant when analyzing LHRH synthesis and protein levels. Hypothalamic LHRH content was assessed by radioimmunoassay, and no change was found after ethanol exposure. Similar results were seen for LHRH mRNA levels as assessed by comparative Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

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LIST OF ABBREVIATIONS

α	alpha
β	beta
BCIP	5-bromo-4chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
cAMP	adenosine 3', 5' - monophosphate
cDNA	complementary deoxyribonucleic acid
D	daltons
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic triphosphate
DTT	dithiolthreitol
EDTA	ethylenediamine tetraacetic acid
FSH	follicle stimulating hormone
g	gram
HEPES	N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid
hnRNA	heteronuclear RNA
k b	kilobases
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
μ Ci	microcurie

μg	microgram
μl	microliter
μM	micromolar
mg	milligram
MgCl_2	Magnesium Chloride
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MOPS	3-(N-morpholino) propanesulfonic acid
NBT	nitro blue tetrazolium chloride
nM	nanomolar
OD.	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethysulfonyl fluoride
RIA	radioimmunoassay
RNA	ribonucleic acid
RT	reverse transcription
SDS	sodium dodecyl sulfate
SSC	sodium chloride/sodium citrate buffer
Taq	thermus aquaticus DNA polymerase
TBE	tris-boric acid-EDTA electrophoresis buffer
UV	ultraviolet
v/v	percent volume in volume
w/v	percent weight in volume
$\times g$	times gravity

CHAPTER I

INTRODUCTION

The physiological effects of ethanol have been a very important area of investigation in the public health sciences for decades. For many of these studies, the adverse effects of ethanol exposure on reproductive function have been reported. It has been shown that both acute and chronic ethanol exposure results in abnormal gonadal steroid levels, loss of gonadal function, and increased incidences of ovulatory failure (Gavaler and Thiel 1987). Reports state that ethanol has a toxic effect upon the hypothalamic pituitary gonadal axis. However, from these studies, it is not clear whether ethanol acts on different components of the hypothalamic pituitary gonadal axis simultaneously or whether its actions are primarily at a single level of the axis.

Acute and chronic ethanol exposure have been shown to result in depressed concentrations of circulating levels of luteinizing hormone (LH) (Cicero et al. 1978; VanThiel et al. 1979; Cicero et al. 1981; Emanuele et al. 1991). The focus of this depression has been on the anterior pituitary and studies using pituitary cells in culture (Pohl et al. 1987; Emanuele et al. 1989). From these studies, it was determined that several points of control could be affected by ethanol. These points were more closely examined by in vivo

studies. These in vivo studies, using Northern blot analysis, showed a decrease in β -LH mRNA in pituitaries of ethanol-treated rats 1.5 and 3.0 hours after ethanol exposure as compared to control (saline) rats (Emanuele et al. 1991). This study also showed a decrease in serum or circulating LH levels from ethanol-treated rats at the same time points. Further studies showed an increase in the intrapituitary content of LH protein. Studies completed in this dissertation attempt to better understand these actions of ethanol at the level of the pituitary. One of the goals of this dissertation was to investigate the hypothesis that ethanol acts directly at the pituitary by altering gene expression of β -LH mRNA.

The second goal of this dissertation was to test the hypothesis that ethanol also acts at the hypothalamus by decreasing Luteinizing-Hormone Releasing Hormone's (LHRH) expression resulting in lower mRNA levels. Several laboratories have studied the effects of ethanol on the hypothalamus and LHRH's secretion from it, but the results have been inconclusive. The evidence, though mainly indirect, suggests ethanol is acting at the hypothalamus by inhibiting LHRH release into the portal blood (Emanuele et al. 1989; Emanuele et al. 1989; Emanuele et al. 1990; Hiney and Dees 1991). Other studies have evaluated LHRH content after ethanol exposure. Chronic studies have found hypothalamic LHRH content to increase after ethanol exposure (Dees and Kozlowski 1984; Rettori et al. 1987). The proposed studies analyze whether or not ethanol manifests its effects on LHRH synthesis.

CHAPTER II

REVIEW OF RELATED LITERATURE

The Reproductive Axis

The Glycoprotein Hormones

Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are anterior pituitary hormones necessary for the initiation and maintenance of reproduction. They are members of a group of glycoprotein hormones which also include thyroid stimulating hormone (TSH) and chorionic gonadotropin (CG). Of the four hormones in this group, three of them, LH, FSH, and TSH are synthesized in the anterior pituitary. CG is synthesized in the placenta. LH and FSH are produced in a subpopulation of cells in the anterior pituitary, the gonadotropes, and TSH is produced in another group of cells, the thyrotrope cells. The glycoprotein hormones are composed of identical α -subunits and dissimilar β -subunits that confer biological specificity to each of the hormones (Gharib et al. 1990).

The rat cDNA and genes encoding the α -subunit, β -LH, and β -FSH have been isolated and characterized (Godine et al. 1982; Chin et al. 1983; Jameson et al. 1984). The rat genes exist as single copies and are located on different chromosomes. Each gene consists of 3-4

exons and 2-3 introns. The rat β -LH gene is relatively small, being about 1.0 kb in size, whereas the α -subunit and β -FSH subunit are significantly larger, 7.7 kb and 3.0 kb, respectively. The rat mRNA sizes are 654 bases, 517 bases, and 1500 bases for the α -subunit, β -LH, and β -FSH, respectively (Gharib et al. 1990).

The lack of a gonadotropin-producing cell line has been a significant obstacle to exploring the regulatory sequences and factors required for expression of the gonadotropin subunits. For the rat α -subunit gene, a sequence known to be a cAMP response element (CRE) in other genes has been identified, but upon further investigation the region did not shown any cAMP responsiveness (Gharib et al. 1990). In other species, several DNA elements have been shown to regulate α -subunit expression. In the mouse two different DNA elements were found to mediate LHRH effects on α -subunit expression (Schoderbek et al. 1992). Also a conserved DNA element in the α -subunit from mouse, human, and horse has been shown to bind a gonadotrope-specific DNA binding protein (Horn et al. 1992). These elements are not found to be present in the rat α -subunit gene.

An estrogen response element has been identified for the rat β -LH gene. This region found between -1388 and -1105 bp can bind to the estrogen receptor and confer estrogen responsiveness (Shupnik et al. 1989). Again, because of the lack of LH-producing cell lines, very few other response elements have been identified.

The rat β -FSH gene is different from the α -subunit and β -LH genes. It contains a long 3' untranslated (UT) region, 1.5 kb in

length (Gharib et al. 1990). Within this region there are 5 highly conserved segments which have been shown to be important in RNA stability (Shaw and Kamen 1986). The removal of this 3'-UT from ovine β -FSH has been shown to enhance expression of β -FSH (Mountford et al. 1992). Whether or not this region is involved in the regulation of β -FSH expression has not been shown.

The circulating and biologically active gonadotropins are composed of the α -subunit protein noncovalently bound to the β subunit. Each of these subunits is internally cross-linked and stabilized by disulfide bonds (Ryan et al. 1988). During translation of the individual subunits, they are processed through the endoplasmic reticulum where two N-linked carbohydrate chains are added to the α -subunit and β -FSH-subunit and one to the β -LH subunit (Hoshina and Boime 1982; Wilson et al. 1990). While in the endoplasmic reticulum, the α and β subunits combine and initial processing of the side chains occur. Modifications to the newly formed heterodimer are then made in the Golgi apparatus. The function of the glycosylation is not known. It has been speculated that it may play a role in facilitating receptor binding and/or clearance of the glycoproteins (Drickamer 1991).

The gonadotropins are regulated at many levels in the endocrine system. Since they play such a central role in normal reproductive functions, it is not surprising to find them under significant and complex control. LH and FSH are regulated in a closed loop system, also referred to as the hypothalamic-pituitary-gonadal axis (Figure 1).

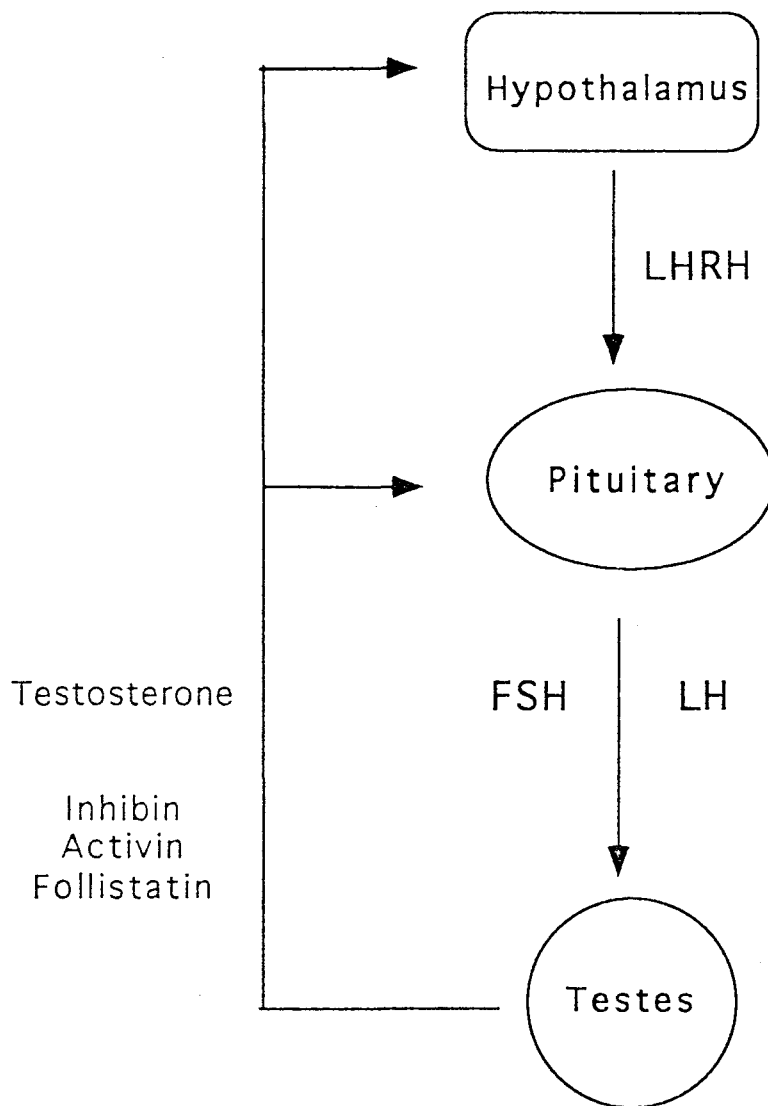


Figure 1 Diagrammatic representation showing the feedback regulation of the male reproductive axis.

Luteinizing Hormone Regulation

Typical regulation of LH secretion begins with the pulsatile secretion of its major regulator, luteinizing hormone-releasing hormone (LHRH), also referred to as gonadotropin hormone-releasing hormone (GnRH), from the hypothalamus. LHRH travels through the portal blood to receptors located on anterior pituitary gonadotrope cells, where LH synthesis and secretion are stimulated. Variations in the amplitude and frequency of LHRH release determines the amount of gonadotropin synthesized and secreted. Increasing the frequency of LHRH pulses increases the frequency and basal secretion of LH (Gharib et al. 1990). Once secreted from the pituitary, LH enters the general circulation and acts on its target organs, the gonads. Steroidogenesis is then stimulated in these organs. Testosterone, feedbacks in a negative manner on the hypothalamus and pituitary, inhibiting LHRH and LH secretion.

When the sex steroids were removed through castration, an increase in the synthesis of the α -subunit and β -LH subunit was observed (Godine et al. 1980; Corbani et al. 1984; Gharib et al. 1990). Testosterone has been shown to have inhibitory effects on α -subunit and β -LH subunit mRNA expression, similar to its effect on secretion and pituitary LH content. The removal of the sex steroids also resulted in an increase in the number of β -LH mRNA containing cells and the amount of β -LH mRNA per cell. It is unclear whether these

effects are manifested at the hypothalamus, the pituitary, or both (Vogel et al. 1986; Gharib et al. 1990).

LHRH has been shown to differentially stimulate α -subunit and β -LH subunit mRNA levels. In vivo studies have shown that the amplitude and pulse frequency were important in the regulation of the stimulatory effect of LHRH on the expression of the two subunits. 7.5 to 8 minutes pulse intervals increased α -subunit expression while not increasing β -LH levels. However, when LHRH was administered with a pulse interval of 30 minutes, expression of both subunits was increased (Gharib et al. 1990).

Follicle-Stimulating Hormone Regulation

FSH is also regulated by sex steroid hormones and by hypothalamic LHRH. FSH regulation by testosterone has been observed to be one of positive regulation. An increase in FSH secretion was seen when pituitary cell cultures were treated with testosterone (Kennedy and Chappel 1985; Gharib et al. 1990). β -FSH mRNA levels have also been shown to be positively regulated by testosterone (Kennedy and Chappel 1985; Gharib et al. 1987; Gharib et al. 1990). Similar to LH, positive regulation was also observed for LHRH. Slow frequencies of LHRH pulses results in an increase in FSH secretion (Pohl et al. 1987) as well as β -FSH mRNA expression (Papavasiliou et al. 1986).

The synthesis and secretion of FSH is regulated differently from that of LH through its regulation by gonadal peptides: inhibin, activin, and follistatin. These are proteins originally isolated from

follicular fluids and have been shown to regulate FSH synthesis and secretion (Carroll et al. 1991; Jakubowiak et al. 1991; Attardi and Winters 1993; Weiss et al. 1993). These peptides have also been found in the pituitary (Roberts et al. 1989), hypothalamus (Ramasharma and Li 1986), brain (Sawchenko et al. 1988), placenta (Petraglia et al. 1987), adrenal glands, bone marrow, and kidney (Meunier et al. 1988).

Inhibin and activin are members of the transforming growth factor- β gene family and are structurally related. Follistatin has no sequence homology to inhibin and activin, but it is able to bind to activin and block its action on FSH secretion (Attardi and Winters 1993). Inhibin and follistatin are negative regulators, while activin is a positive regulator of FSH. Studies have shown all three gonadal peptides to be regulators of β -FSH mRNA (Gharib et al. 1990; Carroll et al. 1991; Attardi and Winters 1993). However, it is not clear whether these hormones act by altering transcription of the β -FSH gene or the stability of the β -FSH transcripts (Carroll et al. 1991; Attardi and Winters 1993; Weiss et al. 1993). Activin had been shown to act at the posttranscriptional level by increasing the stability of β -FSH mRNA (Carroll et al. 1991).

Luteinizing Hormone Releasing Hormone (LHRH)

As stated above, LHRH is a major regulator of reproduction through its control exerted over LH and FSH synthesis and secretion. It also stimulates the recruitment of pituitary cells into the gonadotrope pool (Fink 1988). LHRH is secreted in pulsatile manner

from the hypothalamus exerting its differential effects on LH and FSH synthesis and secretion through changes in its frequency and/or amplitude of its pulses (Leung et al. 1987; Haisenleder et al. 1988; Dalkin et al. 1989).

LHRH-expressing cells are located in several areas of the brain. Using immunocytochemistry, LHRH-expressing neurons have been found in the medial preoptic area, among the the diagonal bands of Broca, in the ventral septum, and in the anterior hypothalamus (Pfaff et al. 1987; Schwanzel-Fukuda et al. 1992). Similar results were obtained using in situ hybridization for the detection of LHRH-expressing cells (Pfaff et al. 1987; Rothfeld et al. 1987). In addition to the these areas of the brain, LHRH protein and gene expression has been detected in the placenta (Seeburg and Adelman 1984; Wierman et al. 1992), gonadal tissue (Bhasin et al, 1983), mammary gland tissue (Harris et al, 1991), the central nervous system (Liposits et al. 1991; Kelley et al. 1993), and the lymphocytes (Azad et al, 1992).

LHRH is a decapeptide which arises from the posttranslational processing of a 92 amino acid precursor protein, prepro-LHRH (Adelman et al. 1986; Wetsel et al. 1988; Kelly et al. 1989). The precursor protein is comprised of 10 amino acids for LHRH, a signal peptide of 23 amino acids, a 3 amino acid sequence necessary for the proper protease cleavage of the precursor protein, and a 56 amino acid sequence, encoding the GnRH-associated peptide, or GAP. GAP has been shown to inhibit the secretion of prolactin from pituitary cells in culture and is also referred to as PIF (Prolactin release-inhibiting factor). Using the same pituitary cell culture system, GAP

was also shown to stimulate both LH and FSH secretion (Nikolics et al. 1985) .

Regulation of LHRH Secretion

LHRH secretion is regulated by many factors. Gonadal hormones, gonadal peptides, endogenous opioid peptides, and catecholamines have all been shown to affect LHRH. Valenca and colleagues (1987) have also demonstrated the ability of LHRH to regulate its own secretion through an ultrashort loop feedback system.

Gonadal hormones have been shown to exert at least two types of negative feedback. First, gonadal hormones act at the level of the hypothalamus to decrease LHRH pulse frequency. Studies related to this effect were performed by removing the gonads of male rats, thereby removing the circulating gonadal hormones, and then sampling the portal blood through push-pull perfusion for LHRH release and the serum for LH content (Levine et al. 1991). When comparing castrated and sham-castrated controls, LHRH pulse frequency and not LHRH pulse amplitude was found to be significantly increased in the castrated group. The second effect gonadal hormones have is at the pituitary with a suppression of responsiveness to LHRH. Strobl and colleagues (1989) demonstrated this using a rat model in which endogenous hypothalamic factors were removed through hypophysectomy and the pulse pattern of LHRH controlled for in castrated and sham-operated animals. LH levels in the castrate group rose steadily reaching a plateau at 18

hours post castration. No change was found in sham-operated controls. Since the amplitude and the pulse frequency of LHRH remained constant during the 18 hours post castration, the increase in LH levels found in castrated rats was concluded to be a result of the inhibition of pituitary responsiveness to LHRH.

Gonadal steroids are thought to also act by altering levels of hypothalamic catecholamines and endogenous opioids (Levine et al. 1991). Endogenous opioid peptides (endorphins and enkephalins) have been shown to inhibit LHRH secretion. Using opiate receptor antagonists, such as naloxone, LHRH pulse amplitude was found to be increased. Opposite effects are seen with the catecholamines, epinephrine and norepinephrine. Using an α -adrenergic receptor antagonist, LHRH pulse frequency was found to be decreased, supporting the hypothesis that catecholamines are stimulators of LHRH secretion.

The gonadal peptides inhibin and activin have also been shown to affect LHRH secretion. Vale and colleagues (1990), using an LHRH secreting cell line, found activin to increase LHRH secretion from these cells. Inhibin did not affect the secretion of LHRH when it was used alone; however, in combination with activin, it was able to partially block the increase of LHRH secretion seen with activin alone (Gonzalez-Manchon et al. 1991).

Regulation of LHRH gene expression has been less defined compared to the secretion of LHRH. The gene and hypothalamic cDNA for LHRH and GAP have been isolated (Adelman et al. 1986). The rat gene is composed of four exons with the second exon

encoding the LHRH peptide. Recently, the LHRH gene promoter has been cloned and a functional analysis completed (Kepa et al. 1992). Two TATA-like regions, located at -19 and -90, and CAAT consensus sequence, located at -56, were found in the sequence. There were no consensus steroid hormone response elements present in the rat sequence. However, 5 halfsites for the ER/TRE response elements were found at -2687, -1479, -1059, -492, and -99. Retinoic acid may be involved in LHRH expression since two consensus sequences for retinoic acid response elements (RARE) were also noted. Furthermore, several consensus sequences for members of the POU-domain family of DNA binding proteins occurred at -1420, -2440 (Pit-1), -2264 (Oct-1), and -154 and -2009 (Tst1) (Kepa et al. 1992).

Studies using an LHRH producing cell line and 5'-deletion analysis found an activation region to be present between -3026 and -1031 and a suppressor region between -1031 and -903 upstream. Four of the 5 consensus sequences for POU-domain family are located within the activation region (Kepa et al. 1992). The functional importance of the individual DNA binding proteins in LHRH expression is not yet known.

LHRH initiates its effects on LH and FSH by binding to specific receptors on the gonadotrope membrane. Concentrations of LHRH receptors have been shown to vary during different endocrine states and throughout development. Receptor number is regulated by LHRH itself as well as by gonadal steroids (Marshall and Kelch, 1986; Conn et al, 1987). How LHRH acts intracellularly is not completely understood. Mobilization of calcium from external stores, G-protein

activation, increased phosphoinositol turnover, calcium calmodulin interaction, and redistribution of protein kinase C (PKC) have all been shown to play a role in LHRH action on gonadotropin release (Conn et al. 1987; Conn 1989). The exact steps of the intracellular activation is not known at this time.

Effect of Castration on the Hypothalamic-Pituitary-Gonadal Axis

Removal of gonadal steroids by castration leads to a prompt increase in plasma gonadotropin levels (Badger et al. 1978; Gharib et al. 1990; Kitahara et al. 1990). Increase in synthesis of gonadotropin subunits (α , β -LH, and β -FSH) after castration was first reported by Godine, et. al. (1980). More extensive studies were done a few years later. In these studies with male rats, there were gradual rises in α -subunit (3-5 fold) and β -LH subunit mRNA levels following castration, reaching plateaus approximately 3 weeks post castration (Corbani et al. 1984; Gharib et al. 1990). However, β -FSH mRNA levels increase 4-fold by 7 days post castration, decline 1.5 fold by 28 days post castration and rise again 4-fold 90 days post castration. There have been conflicting results on the effect of castration on hypothalamic LHRH synthesis and secretion. Park and colleagues (1988) observed a decrease in LHRH mRNA 25 days after castration. A similar decrease in LHRH mRNA after castration was reported using in situ hybridization (Zoeller et al. 1988). Using in situ hybridization, Rothfeld (1987) was unable to detect a change in LHRH mRNA expression after castration.

Ethanol and the Reproductive Axis

Ethanol and the Testes

It has been shown many times that serum testosterone levels fall after ethanol exposure (Mendelson et al. 1978; Cicero et al. 1981; Mello et al. 1985). Salonen and Huhtaniemi (1988) have shown that ethanol exerts effects directly on the gonad by decreasing testicular content of LH receptors. Male rats were given ethanol (2.2g/kg per day) for one week and the number of LH receptors in the testes were evaluated at that time. LH receptor levels were significantly decreased after the ethanol exposure (Salonen and Huhtaniemi 1988). Testicular LH receptors were also found to be significantly reduced after chronic ethanol exposure (ethanol diet for 5 weeks) (Salonen and Huhtaniemi 1990). Additional evidence for direct action of ethanol on the gonads was provided by studies showing ethanol inhibiting human chorionic gonadotropin (HCG)-stimulated testosterone secretion and production in both chronic and acute exposure (Cicero et al. 1983; Esquifino et al. 1989).

Many in vitro studies have demonstrated ethanol's toxic effect on testosterone production (Santucci et al. 1983; Orpana et al. 1990). It has been suggested that a metabolite of ethanol, acetaldehyde, is the inhibitor of testosterone production. Several studies have shown acetaldehyde is a strong inhibitor of testosterone and that these inhibiting effects are reversible when 4-methylpyrazole, an inhibitor of ethanol metabolism, is added to the cells (Cicero et al. 1981; Murono 1983; Orpana et al. 1990). These studies suggest that

ethanol is directly acting at the testes altering testosterone production.

Ethanol and the Pituitary

Ethanol and Serum LH Levels

While a decrease in testosterone should result in release of the negative feedback loop on LH synthesis and secretion, a decrease in serum LH levels is seen. The decrease in serum LH levels has been demonstrated whether the alcohol exposure is acute (Cicero et al. 1978; Cicero et al. 1981) or chronic (VanThiel et al. 1979; Esquifino et al. 1989) in intact or castrated male rats. In the castrate model, the time of ethanol exposure after castration was demonstrated to be of importance. Cicero et. al. (1990) showed serum LH levels to be significantly decreased after acute ethanol exposure, if given up to two weeks post-castration. At 2 weeks post-castration a significant increase in serum-LH levels was seen, followed by a significant decrease (compared to controls) in serum LH levels at 3 weeks post-castration.

Numerous studies have attempted to determine if ethanol's effect on LH is at the level of the pituitary. Again, the results are inconsistent but support the theory that ethanol has a direct inhibitory effect on the pituitary gland. The effect of ethanol on the pituitary gland was studied by measuring ethanol's ability to influence LHRH-induced LH secretion. The results of in vivo experiments depended on whether ethanol was acutely or

chronically administered. In acute ethanol exposure, ethanol did not inhibit LHRH-stimulated LH release (Cicero et al. 1978). Whereas, in chronically exposed rats, ethanol inhibited LHRH-stimulated LH release (VanThiel et al. 1979; Chung 1989). In in vitro studies using dispersed cultured cells from the anterior pituitary, several laboratories have shown that ethanol significantly inhibits LHRH-stimulated LH release (Pohl et al. 1987; Emanuele et al. 1989). These studies suggest ethanol's effect is mediated by alterations in the pituitary's sensitivity to LHRH stimulation (Purohit 1993).

Ethanol and Intrapituitary LH Content

Ethanol's effect on pituitary LH content are inconsistent. Salonen and Huhtaniemi (1990), using a chronic ethanol model (ethanol diet for five weeks), found pituitary LH content to be significantly increased compared to control animals. Results, using an acute ethanol model (i.p. injection 3g/kg) (Emanuele et al. 1991), found that pituitary LH content was significantly increased 1.5 hours post injection; levels returned to control values by 3 hours post injection. However, Cicero and colleagues (1990) did not find any significant changes in pituitary LH content, following an acute ethanol treatment.

Ethanol and α - and β - LH mRNA Transcription

The effect of ethanol on α - and β -LH transcription reportedly has differential effects on the two subunits. After acute in vivo ethanol exposure (single i.p. injection), β -LH mRNA was significantly decreased 1.5 and 3.0 hours post-injection (Emanuele et al. 1991).

This suppression returned to control levels by 24 hours post-injection. The common α -subunit mRNA was unaffected by acute ethanol exposure (Emanuele et al. 1991). Similar effects were seen for α -subunit mRNA in chronically fed ethanol rats, where no change was reported. However, using this chronic model, β -LH mRNA was significantly higher in ethanol-fed rats compared to pair-fed and chow-fed animals (Salonen et al. 1992).

Ethanol and Follicle-Stimulating Hormone

There have been very few studies of the effect of ethanol on FSH. Salonen and Huhtaniemi (1990) found a decrease in serum FSH levels in male rats after a chronic ethanol diet. Salonen (1992) later showed a significant fall in β -FSH mRNA in alcohol-treated animals when compared with pair-fed controls but not when compared to chow-fed animals. In both studies, no change in pituitary FSH content was reported. The lack of depletion, in fact, is important and may imply block release.

To summarize, ethanol has differential effects on the pituitary depending on the gonadotroph. Acute and chronic ethanol exposures result in a decrease of LH and FSH in the serum. The effect of acute ethanol exposure on pituitary protein levels increase for LH and do not change for FSH.

Ethanol and the Hypothalamus

The effect of ethanol on the hypothalamus has been investigated by measuring hypothalamic LHRH content, portal blood LHRH concentration, and naloxone-induced release of LH to plasma. It has been suggested that ethanol exerts its effects on the hypothalamus by increasing synthesis and/or release of opioids, thus decreasing LHRH release from the hypothalamus. Naloxone, an inhibitor of opioids and therefore a stimulator of LHRH, was used to study this possibility. Cicero (1983) showed ethanol was able to partially block LH response to naloxone. Ethanol's ability to block the stimulation of LHRH release by naloxone indicates that ethanol has an effect on the hypothalamus through a mechanism involving opioids. However, this study does not rule out the possibility that naloxone is also acting at the level of the pituitary and increasing LHRH synthesis and release of opioids found to be present there.

Hypothalamic LHRH content in rats after ethanol exposure varies. Dees and Kozlowski (1984) found that after 2 days of ethanol exposure (1.25 g/kg injected every 4 hours), hypothalamic LHRH concentrations increased while serum LH levels were significantly decreased. This finding was seen in both intact and castrated male rats. This effect was also seen in adult female rats (Rettori et al. 1987). These studies suggest an effect of ethanol directly on the hypothalamus possibly through a reduced rate of LHRH release. However, the studies on the effect of ethanol on LHRH release have been inconclusive. When looking at portal blood levels of LHRH after ethanol exposure (single i.p. injection), Ching and colleagues (1988)

found a significant decrease. A similar decrease was found in vitro with ethanol decreasing stimulated but not basal LHRH release from the hypothalamus (Hiney and Dees 1991). However, ethanol was shown in numerous in vitro studies to have no effect on either stimulated or basal hypothalamic LHRH release (Emanuele et al. 1989; Emanuele et al. 1989; Emanuele et al. 1990).

In summary, studies using chronic ethanol exposure have found an increase in hypothalamic LHRH content. The effect of ethanol on LHRH release is unclear. In vivo studies demonstrated a decrease in LHRH release from the hypothalamus. In contrast, in vitro studies repeatedly found ethanol to have no effect on LHRH release.

Proposed Studies

Ethanol has been shown to be deleterious to the hypothalamic-pituitary-gonadal axis. Much of the research has focused on circulating reproductive hormone levels and secretory changes of these hormones from cells after ethanol exposure. This dissertation focused on the effect that ethanol has on the molecular level of the reproductive axis. The purpose of this dissertation was to investigate the hypothesis that ethanol acts at the level of the pituitary by altering gene expression of β -LH mRNA. A second hypothesis investigated was that ethanol also acts on the hypothalamus by decreasing LHRH's expression in lowering mRNA levels.

CHAPTER III

MATERIALS AND METHODS

Animals

All animals used were adult male Sprague-Dawley rats obtained from Harlan, Indianapolis, IN. The animals were housed in individual cages with 12 hour light: 12 hour darkness regime at 22-24° C. In order to enhance α -subunit, β -LH, and β -FSH mRNA and serum LH and FSH levels, the rats were surgically castrated. Castration was carried out using light pentobarbital anesthesia (40 mg/l x gm). The rats were allowed to recover for two weeks. All of the experiments were completed between 15 and 17 days after castration.

Acute Ethanol (EtOH) Administration

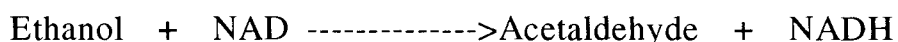
All animals were either given a single intraperitoneal (i.p.) injection of ethanol or saline. The EtOH treated group was given an injection of ethanol at a concentration of 3g ethanol/ kg body weight. A 25% ethanol solution was used and 1ml of this solution was given for every 80 g of body weight. Control animals were given an injection of 0.9% saline (1 ml/80 g of body weight).

Tissue Removal

Animals were sacrificed by decapitation at time points of 1.5 hr, 3.0 hr, and 24 hr after the i.p. injection . The brain was removed from the cranial cavity, and the pituitary stalk severed. The pituitary was removed whole. The anterior pituitary was then separated from the posterior pituitary. The hypothalamus and preoptic area were dissected from the brain using a scalpel. Each tissue was placed in a microcentrifuge tube in a dry ice methanol bath (-70°C). The tissues were stored at -70°C until needed.

Blood Ethanol Determination

Blood samples were taken from the trunk blood at the time of decapitation. Serum was obtained by centrifuging for 15 minutes at $2000 \times g$. The blood EtOH concentrations were determined using an enzymatic kit (Sigma # 330-1; St. Louis, MO). The kit is based on the following reaction catalyzed by alcohol dehydrogenase (ADH):



The reduction of nicotinamide adenine dinucleotide (NAD) to NADH results in an increase in absorbance at 340 nm. This increase in absorbance is directly proportional to the alcohol concentration in the sample.

Three milliliters of glycine buffer (0.5mol/L, pH 9.0) was added to each cold NAD-ADH assay vial. The vial was then capped and mixed by inversion. Once the vial was at room temperature, 10 μl of serum was added, mixed by inversion, and incubated at room

temperature for 10 minutes. The sample was transferred to a cuvette and covered. The absorbance was recorded at 340 nm.

Protein Determination

The amount of protein was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA). This assay is based on the observation that the absorbance for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (Reisner et al 1975). To carry out the procedure, 0.1ml of bovine serum albumin (BSA) standards (20-140 ug) or sample were placed in 5 ml of dye reagent supplied in the kit. The samples were vortexed, placed at room temperature for five minutes, and the OD₅₉₅ versus a reagent blank were read. A standard curve was made by, plotting OD₅₉₅ versus the concentration of BSA standards. Unknown protein values for the samples were then determined using this standard curve.

RNA Isolation from Tissue

Total RNA was isolated by homogenizing the tissue in 500 µl of guanidinium-thiocyanate solution (GIT) (4M guanidine isothiocyanate, 25mM sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol) in a microcentrifuge tube (Emanuele et al. 1991). The sample was then placed on ice for 5 minutes. After this time, 50 µl of 2 M sodium acetate (NaOAc) (pH4.0), 500 µl of phenol and 100 µl of chloroform: isoamyl alcohol (49:1) were added, with mixing after each addition, and incubated on ice for 15 min. The sample was

then centrifuged at 4 °C at 10,000 x g for 15 min. The aqueous (top layer) phase was removed and mixed with an equal volume of isopropyl alcohol to precipitate the RNA. This mixture was placed in a dry ice methanol bath (- 70 °C) for 30 minutes. After centrifuging the samples (10,000 x g for 15 min.), alcohol was removed and the pellets were resuspended in 100 ul of GIT solution. The RNA was precipitated as above, pelleted by centrifugation, washed with 70% ethanol, resuspended in diethyl pyrocarbonate (DEPC) treated water, and stored at - 70 °C.

Northern Blot Analysis

Total RNA was electrophoresed on an agarose (1.4%) formaldehyde gel which was then soaked in 10 X sodium chloride/sodium citrate buffer (SSC) (1.5M NaCl, 0.15 M Na Citrate, pH 7.0) for two twenty minute periods. The RNA was transferred to Nytran (Schleicher and Schuell) by capillary action overnight. Following transfer, RNA was crosslinked to the membrane using an UV Crosslinker (Stratagene).

The membranes were pre-hybridized for at least one hour at 42 °C in 50% formamide, 5X Denhardt's solution (1X Denhardt's stock solution contains 0.02% polyvinylpyrrolide, 0.02% bovine serum albumin, and 0.02% Ficoll 400), 0.01% sodium dodecyl sulfate (SDS), 10 mM NaCl, 0.1 mM NaPO₄ (pH 6.5), 0.001% pyrophosphate, and 0.025 mg/ml salmon sperm (heat denatured). Labeled probe (see below) was then added and hybridized overnight with the membrane at 42 °C. After hybridization, the membranes were

washed three times at high stringency (0.2 X SSC, 0.5% sodium dodecyl sulfate (SDS) at 65 °C) to remove excess labeled probe. The filter was blotted dry and exposed to film at - 70 °C (Kodak Hyperfilm MP). When the filters were to be rehybridized with a different probe, they were stripped of the hybridized signal using a stripping buffer [0.1 % SSPE (1X SSPE contains 150 mM sodium chloride, 10 mM sodium phosphate, and 1 mM EDTA) and 0.25% SDS]. The filters were washed three times using boiling buffer and exposed to film to verify that all the labeled probe had been removed.

Densitometric Analysis

Densitometric scans were performed using the Tri Microscan System (Technology Resources, Inc., Nashville, TN). The two-dimensional gel analysis program was used to scan autoradiograms. The appropriate band was identified and scanned. The number given as the volume of the band was used as the optical density value.

Normalization of Blots

Optical density values for Northern blots probed with cDNA for 28 S ribosomal RNA were collected. Normalization was achieved by calculating ratios of the optical density values for 28 S of a specific lane relative to the lane with the greatest optical density value for 28 S. These values were then used as a correction factor for differences due to unequal loading of RNA onto the gel. The arbitrary densitometer units (A.D.U.) were calculated by taking the

optical density value of a lane for a hormone of interest and multiplying it by the correction factor for that lane.

Labeling of cDNA Probes

The probes used were obtained from following sources:

1) α and β LH cDNA - Dr. William Chin of Harvard Medical School, Boston, MA

2) β FSH cDNA - Dr. Kelly Mayo of Northwestern University, Chicago, IL

3) LHRH cDNA - Dr. John Adelman of the Vollum Institute, Portland, OR

4) 28S rRNA- Dr. Sully Reed of University of Missouri, Kansas City, MO

The cDNA insert was isolated and labeled by the random hexamer primer method (Feinberg and Vogelstein 1984).

Approximately 25 ng of DNA was added to deionized distilled water and denatured at 100 °C for 3 min. and rapidly chilled on ice. To the sample, 10 μ l labeling buffer (250mM Tris-HCL, pH 8.0, 25mM MgCl₂, 10mM DTT, 1mM Hepes, pH 6.6, 27 A₂₆₀ U/ml pd (N)₆ hexamers (Pharmacia)), 2 μ l acetylated BSA (1mg/ml), 2 μ l dNTP's (1.5mM each ATP, GTP, CTP, and TTP), 50 μ Ci alpha ³²P-dCTP (3000Ci/mmol - New England Nuclear), 5 U of Klenow, and deionized distilled water to a volume of 50 μ l were added. The reaction was incubated at room temperature for 1 h, denatured by heating at 100°C for 2 min. and chilled immediately on ice. Two microliters of 0.5 M EDTA and 48 μ l of deionized distilled water were then added. Unincorporated ³²P -

dCTP was removed by centrifugation through a spin column containing Sephadex G-50.

Subcellular Fractionation

Subcellular fractionation was carried out as published using discontinuous sucrose density centrifugation (Trifaro and Duerr 1976). Five pituitaries were homogenized in 2.5 ml of ice cold 0.3M sucrose (pH 7.0) and centrifuged at 20,000 X *g* for 20 min. The pellet was resuspended in 0.3 M sucrose and layered onto a discontinuous sucrose gradient (top to bottom: 0.8 M, 1.0 M, 1.2 M, 1.4 M, and 1.6 M sucrose). The samples were centrifuged at 113,000 X *g* for 70 min. The crude Golgi/ER fraction (0.8 M and 1.0 M interface) was identified by the banding pattern and enzyme activity analysis. This fraction was removed using a glass pipet, brought up to a final concentration of 1.1 M sucrose, and placed onto a second discontinuous density gradient (top to bottom: 1.25 M, 1.3 M, 1.4 M sucrose). A layer of 0.5 M sucrose was then carefully placed on top of the sample. The gradient was then centrifuged at 100,000 X *g* for 90 min. One milliliter fractions were removed from the top of the tube and assayed for galactosyltransferase activity, a characteristic marker for Golgi apparatus (Trifaro and Duerr 1976). The fraction identified as containing Golgi apparatus was confirmed by electron microscopy. Western blot analysis (see below) was carried out on these fractions.

Galactosyltransferase Assay

The activity of galactosyltransferase was estimated from the increase in rate of UDP-galactose hydrolysis which occurs in the presence of a suitable acceptor (Trifaro and Duerr 1976). Samples were incubated for 60 min. in a total volume of 80 μ l. The assay mixture contained 6 μ moles sodium cacodylate, 3 μ moles 2-mercaptoethanol, 3 μ moles MnCl_2 , 0.05 μ moles UDP- ^{14}C -galactose and 0.6% Triton x-100. The reaction was terminated by the addition of 6 μ moles EDTA in 20 μ l deionized distilled water. The tubes were cooled on ice and the mixture passed through a Dowex 2x-8 column. The column was washed with deionized distilled water and the effluents are collected into scintillation vials containing scintillation liquid. The radioactivity was measured using a scintillation counter. Control tubes containing all of the above substances, except N-acetylglucosamine, were included in the assay.

Nuclear Run-Off Assay

Three pituitary were pooled and homogenized in cell lysis buffer (0.25 M sucrose, 55 mM Hepes (pH 7.4), 5 mM MgCl_2 and 0.1% Triton x-100.. The nuclei were isolated by placing the above homogenate on a 0.5 M sucrose cushion and a centrifugation at 6000 rpm for 10 minutes. The pelleted nuclei were resuspended in a buffer containing 200 μCi ^{32}P -UTP (Amersham, Arlington Heights, IL), 0.5 mM each rGTP,rATP,rCTP, 17% glycerol, 60 mM HEPES, (pH 7.0), 2 mM DTT, 25 mM ammonium sulfate, 3 mM magnesium acetate, 3 mM MnCl_2 , and 5 mM sodium fluoride. After the reaction

had been incubated at 37° C for 30 minutes, the newly synthesized RNA (labeled) was precipitated using the same procedure stated above for the Northern blot RNA. The labeled RNA was then hybridized to a Nytran filter containing the LH cDNA and the necessary control plasmids for 72 hours at 42° C. After hybridization, the filters were washed with increasing stringency (6X SSC and 0.2% SDS for 20 minutes; 2X SSC and 0.2% SDS for 20 minutes; 0.2% SSC and 0.2% SDS for 20 minutes). The filters were then exposed to X-ray film (Hyperfilm MP).

Staining and Electron Microscopy of Golgi Fraction

The negative staining procedure was performed on a drop of the sample (1:10 dilution) placed on a carbon-coated collodion filmed copper grid (Trifaro and Duerr 1976). A drop of 2% phosphotungstic acid (pH 7.2) was applied to the grid. The excess stain was immediately removed by touching the edge of the grid with filter paper. Electron microscopic examination of the samples was carried out by Dr. John McNulty, Department of Anatomy and Cell Biology, Loyola University of Chicago.

LHRH Radioimmunoassay (RIA)

LHRH RIA was conducted by using antiserum (LHRH-CRR11B73) supplied by Dr. Victor Ramirez, Department of Physiology, University of Illinois. Each assay tube contained 100µl of either rabbit anti-rat LHRH (1:20,000 dilution) or buffer (0.1M phosphate buffered saline (PBS), 0.05M EDTA, 1% normal rabbit serum), 200 µl

of standard LHRH (Sigma, St. Louis, MO) or unknown, and 100 μ l of 125 I LHRH (10,000 cpm/tube). Iodination was carried out by the Chloramine T method. The mixture was incubated at 4 $^{\circ}$ C for 72 hrs. Two hundred microliters of goat anti-rabbit IgG (Pel Freeze Biologicals) was added to each tube and incubated another 4 hrs. At the end of this incubation, 1.5 ml of ice cold 95% ethanol was added and incubated at 4 $^{\circ}$ C for 20 min. Precipitate was collected by centrifugation at 2000 X g for 30 min., and counted for one minute each on a gamma counter. Assay sensitivity was 7.9 pg/ml and interassay coefficient of variation was 5%. The intraassay coefficient of variation was 9%.

LH Radioimmunoassay (RIA)

The LH RIA was conducted using materials contributed by the National Hormone and Pituitary Program (NIADDK). Each assay tube contain 200 μ l of either rabbit anti-rat LH (1:10,000 dilution) or buffer (0.1M PBS, 0.05M EDTA, 3% normal rabbit serum), 200 μ l of standard (NIADDK-rat-LH-RP-2) or unknown, and 100 μ l of 125 I LH (15,000 cpm/tube). Iodination was carried out by the Chloramine T method. Pituitary aliquots were diluted 1:2000 and 1:4000 before being placed in the assay tube. The mixture was incubated at room temperature for 72 hrs. After this incubation, 200 μ l of goat anti-rabbit IgG (Pel Freeze Biologicals) was added to each tube and incubated another 4 hrs. After 4 hrs., 1 ml of ice cold saline was added and the tubes centrifuged at 2000 X g for 30 min. at 4 $^{\circ}$ C to collect the precipitate. The samples were counted for one minute

each on the gamma counter. Assay sensitivity was 157 pg/ml and inter assay coefficient of variation is 9%. The intraassay coefficient of variation was 5%.

FSH Radioimmunoassay (RIA)

The FSH RIA was conducted using materials contributed by the National Hormone and Pituitary Program (NIADDK). Each assay tube contain 200ml of either rabbit anti-rat FSH (1:10,000 dilution) or buffer (0.1M PBS, 0.05M EDTA, 3% normal rabbit serum), 200 ml of standard or unknown, and 100 ml of ^{125}I FSH (15,000 cpm/tube). Iodination was carried out by the Chloramine T method. Pituitary aliquots were diluted 1:2000 and 1:4000 before being placed in the assay tube. The mixture was incubated at room temperature for 72 hrs. After this incubation, 200 ml of goat anti-rabbit IgG (Pel Freeze Biologicals) was added to each tube and incubated another 4 hrs. After 4 hrs., 1 ml of ice cold saline was added and the tubes centrifuged at 2000 X g for 30 min. at 4° C to collect the precipitate. The samples were counted for one minute each on the gamma counter. Assay sensitivity was 78 pg/ml and interassay coefficient of variation is 12.9%. The intraassay coefficient of variation was 3.7%.

Overexpression of Fusion Proteins in *E. coli*

The expression vector containing LH was constructed by Dr. Mark Kelley using the polymerase chain reaction (PCR). The vector used was pGEX-3X (Pharmacia LKB). The pGEX-3X vector with insert produced a fusion protein with glutathione S-transferase (GST) at the

amino end of the protein with a Factor Xa cleavage site between the GST portion of the fusion protein and LH protein.

An overnight culture of LB broth (5 g NaCl, 10g tryptone, 5 g yeast extract/ 1 L) containing ampicillin (100µg/ml) was diluted 1:10 into fresh LB broth. The culture was grown at 37 °C with shaking for 1 h. Isopropyl-β-D-thiogalactopyranoside (IPTG), an inducing agent, was added to the culture (final concentration 0.1 mM) and the culture was kept at 37 °C for another 2-4 hours. The cells were collected by gentle centrifugation (500 X g) for five minutes and resuspended in buffer A (20 mM Tris-HCL, pH 7.5, 20% sucrose, 1 mM EDTA) for isolation of the overexpressed protein. Crude extracts were run on sodium dodecyl sulfate (SDS) - polyacrylamide gels followed by staining to determine the extent of overexpression and molecular weight.

Purification of Antigen for Antibody Production

The suspension of the fusion protein was added to 2X SDS-gel sample buffer (120 mM Tris-HCL, pH 6.8, 20% glycerol, 70 mM SDS, 2 % 2-mercaptoethanol, 0.5 mg Bromophenol Blue) and boiled for five minutes. The sample was then centrifuged for five minutes at 12,000 X g. Approximately 20 µl was loaded per well on a 1.5 mm thick 12% SDS polyacrylamide gel. The gel was electrophoresed at 100 volts for 3 hours in 1X SDS running buffer (25 mM Tris-HCL, pH8.0, 200 mM glycine, and 0.1% SDS). The gel was stained with Coomassie Blue (0.05% Coomassie Blue, 20% methanol, 10% acetic acid) for 30 min. and destained with several washes of 20% methanol

and 10% acetic acid. The band containing the fusion protein was cut out of the gel and placed into dialysis tubing containing 1X SDS running buffer. The tubing was then placed in a horizontal gel apparatus and the proteins were electroeluted at 30 mA for two hours or until the blue dye had run out of the gel slices. After elution, the gel slices were removed and the tubing containing the protein solution was dialyzed against PBS (150 mM NaCl, 10mM NaPO₄, pH 7.4) overnight.

Production and Purification of Antibodies

The overexpressed protein (antigen, 100 µg) was emulsified in Freund's complete adjuvant and injected into a female rabbit. After three weeks, another 100 µg of antigen was injected into the rabbit, following emulsification in Freund's incomplete adjuvant. Boosts of antigen were given every three weeks. At the time of each antigen boost, the rabbit was bled from the ear to determine the titre of antibody by Western blot analysis.

The antibody was purified using immunopurification following the protocol published by Maniatis et al. (1989) (Maniatis et al. 1989). Overexpressed protein was electrophoresed on SDS-polyacrylamide gels and electroblotted onto nitrocellulose with 150 mA of electrical current. After incubating the filters in blocking buffer (see Western Blot Analysis) for 1 h at room temperature, sera from the rabbit was added to the filters. The filters were incubated overnight at 4^o C with gentle shaking on a rotator. The sera was removed the next day and saved for further purifications. The filters

were rinsed in 1X TBST (see Western Blot Analysis) three times for 20 min. each. One lane was removed to determine the position of the antigen on the blot using an alkaline-phosphate conjugated anti-rabbit IgG antibody. The area containing the antigen was cut out of the filter. Antibody was removed from the strip by layering elution buffer (0.2 M glycine/pH 2-8, 1mM EGTA) on it and then incubating it for 30 min at room temperature with gentle shaking. The elution buffer was collected, neutralized with 0.1 volume of 1 M Tris-HCL, pH 9.5 and made to a final concentration of 1 x PBS. The antibodies were stored at 4 °C.

Western Blot Analysis

Tissues were homogenized in buffer [10 mM Tris-HCl, pH7.5, 1% SDS, 0.2% Triton X-100, 0.2% nonidet P-40, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF;(300 µl /pituitary)]. Protein samples were added to equal volumes of 2X loading buffer (120 mM Tris-HCl, pH 6.8, 70 mM SDS, 20% glycerol and 2% 2-mercaptoethanol) and heated at 100 °C for 5 min., followed by a five min centrifugation at 12,000 X g . The samples were then loaded on a 12% SDS-polyacrylamide gel and electrophoresed at 100-150 volts in 1X running buffer (25 mM Tris-HCL, 200 mM glycine and 0.01% SDS) for about 2 hours. The gels were then placed in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% methanol) for 30 min. Proteins were electroblotted onto 0.2 micron nitrocellulose (Schleicher and Schuell) at 150 mA of current for 1 h. The filters were soaked in blocking solution [1 mM Tris-HCl, pH 8.0, 15 mM NaCl, 0.005 % Tween 20 (1X

TBST) plus 5% powdered milk]. The primary antibody (1:1000 dilution) was added to the filters and incubated overnight at 4 °C. After washing the filters in 1X TBST three times for 10 min. each, secondary antibody was placed in blocking buffer and incubated with the filter for 2-3 hours at room temperature. Two different types of second antibodies were used. If the anti-rabbit alkaline-phosphatase conjugate was used, the filters were washed as above and developed in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂) containing 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and Nitro blue tetrazolium chloride (NBT). The development was stopped by placing the filter in stop solution (20 mM Tris-HCl, pH 8.0 and 5 mM EDTA). If the secondary antibody was ¹²⁵I-labeled anti-rabbit-IgG (Amersham; Arlington Heights, IL), the filter was washed as above, blotted dry, and exposed to film (Hyperfilm-MP).

5'-End Labelling of DNA Oligonucleotides

Oligonucleotides (NBI; Plymouth, MN) were resuspended in deionized distilled water at a concentration of 1 pmol/μl. The 5' end of the oligo was labeled by the transfer of the gamma ³²P from ATP to a 5' terminus of the oligo by bacteriophage T4 polynucleotide kinase (Gibco/BRL; Gaithersburg, MD) according to Maniatis *et al.* (1989) (Maniatis et al. 1989). In a final volume of 10 μl, 10 ng of oligonucleotide was added to 1 X T4 kinase buffer (0.05M Tris-HCl, pH 7.6, 0.01 M MgCl₂, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, pH 8.0), 50 μCi gamma-³²P-ATP, and 8 units of T4 polynucleotide

kinase. The reaction mixture was placed at 37 °C for 30 min. The reaction was stopped by heating the tube at 90 °C for two minutes and then placing it on ice. Purification of the labeled oligonucleotide was performed by gel electrophoresis. An equal volume of 2X loading dye [90% formamide, 0.5 X TBE (5X Tris-HCl, Boric Acid, EDTA), 0.1% Bromophenol blue, 0.1% xylene cyanol) was added to the labeled probe and heated at 90 °C for 10 min. The probe was electrophoresed on a 15% polyacrylamide gel. The labeled probe band was cut out of the gel and placed in a tube with gel elution buffer (0.5 M NH₄OAc, 1 mM EDTA, 0.1% SDS) overnight at 37 °C. The gel pieces were removed the next day and the probe was ready to be used in the S1 nuclease protection assays.

S1 Nuclease Protection Assay

Total RNA was isolated from three pooled pituitaries and resuspended in DEPC water (see RNA Isolation). A reaction mixture was made containing 80% formamide, 1X S1 hybridization buffer (4M NaCl, 0.4M Pipes, 0.2 M EDTA), 5 µg tRNA, 1 µl of labeled probe, and RNA to a total volume of 100 µl. The mixture was heated at 70 °C for 15 min. and immediately placed at 48 °C for 12-16 hours. Following this incubation, 300 µl of S1 digestion buffer (66 mM NaOAc, 0.3 M NaCl, 4.0 mM ZnSO₄) and 100 U of S1 nuclease was added and incubated at 37 °C for 60 min. The digestion was stopped by addition of 200 µl of phenol and 200 µl of chloroform: isoamyl alcohol (49:1). The mixture was centrifuged at 4 °C for 10 min. The aqueous layer (top) was removed and 1 ml of 100% EtOH

was added to precipitate the protected fragments. The tube was placed in a dry ice and methanol bath for 15 min and then centrifuged at 12,000 x g for 15 min. at 4 °C. After the ethanol was aspirated, the pellet was dried and resuspended in deionized distilled water. Loading dye (90% formamide, 0.5% TBE, 0.1% Bromophenol Blue, 0.1% Xylene cyanol) was added and samples were heated to 90 °C for 10 min. The sample were electrophoresed on a 15% polyacrylamide gel in 1X TBE buffer at 150 V until the bromophenol blue dye had reached the end of the gel. The gel was then placed on Whatman paper, cellophane placed over it, and exposed to film.

Polysome Distribution Analysis

Five pituitaries were homogenized in 1 ml of HKM buffer (20 mM HEPES, pH 7.6, 100 mM KCl, 20 mM MgCl₂, 0.5% Triton X-100, 3 mM 2-mercaptoethanol, 300U/ ml RNAsin (Promega, Madison, WI) and centrifuged at 12,000 X g for 10 min at 4 ° C to pellet nuclei. The supernatants were overlaid onto a linear sucrose gradient 10% -40% (wt/wt) made in HKM buffer with a 60% (wt/wt) sucrose cushion (Kleene et al. 1984; Murphy et al. 1992). Gradients were centrifuged using a Beckman SW41 rotor for 105 min. at 41,000 r.p.m. After centrifugation, gradients were unloaded manually into fractions (1-10) and the absorbance at 254 nm was determined. SDS was then added to a final concentration of 0.5% to each of the fractions. Each fraction was extracted with phenol:chloroform (1:1) twice. The fractions were ethanol precipitated with 2 vol. 100% ethanol, and 10 µg tRNA. The pellets were resuspended in TE (10

mM Tris-HCl and 1 mM EDTA) with 0.1% SDS. Northern blot analysis was then carried out on the fractions. As a control, equivalent supernatants were prepared and centrifuged in sucrose gradients in buffers in which the MgCl₂ was replaced by EDTA (10 mM).

Reverse Transcription Reaction

Four micrograms of total RNA isolated from the hypothalamus was added to a microcentrifuge tube and the volume was brought to 13 µl with DEPC treated water. Oligo dt (100 pmol, BRL; Gaithersberg, MD) was added to the tube and the mixture was heated at 70 °C for 10 min. The tube was quickly chilled on ice. To the mixture, 50 mM Tris-HCl (pH8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 2 mM of each deoxynucleotide triphosphate (dTTP, dGTP, dATP, dCTP) and Superscript Reverse Transcriptase (200 U, BRL; Gaithersberg, MD) was added to a final volume of 20 µl. The mixture was incubated at room temperature for 10 min and then at 42 °C for 50 min. The reaction was terminated by heating at 95 °C for 5 min and then placed on ice. RNase-H (2 U, BRL; Gaithersberg, MD) was added, and the resulting solution was incubated for 20 min at 37 °C. The resulting cDNA was stored at 4 °C.

Polymerase Chain Reaction

The oligonucleotides used for the PCR reaction are listed below.

LHRH 5': 5'-CACTATGGTCACCAGCGGGG-3'

LHRH 3': 5'-AGAGCTCCTCGCAGATCCCTAAGA-3'

H3.3 5': 5'-GCAAGAGTGCGCCCTCTACTG-3'

H3.3 3': 5'- GGCCTCACTTGCCTCCTGCAA-3'

Five microliters of the reverse transcription reaction were diluted to a final volume of 100 μ l in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.01% Tween-20, and 0.01% Nonidet P-40, 2 μ M of each oligonucleotide, 2 μ Ci ³²P-dCTP (3000 Ci/mmol, NEN; Boston, MA) and 2 U Taq polymerase (BRL;Gaithersberg, MD)(Kelley et al. 1993). The polymerase amplification was carried out using an Eppendorf MicroCycler (Fremont, CA) for 22 cycles of amplification (94 °C, 30 sec; 60 °C, 1 min; 72 °C, 2 min), followed by 72 °C for 10 min. Ten microliters of the sample was electrophoresed on a 2% agarose gel, transferred to Nytran (Schleicher and Schuell) and exposed to film. The expected PCR products were 375 bp for LHRH and 213 bp for H3.3.

Statistical Analysis

All experiments were analyzed by one-way analysis of variance (ANOVA) to determine if there were any differences among the means of the groups being compared. Bonferroni test was used as a *post hoc* analysis. A p value < 0.05 was considered significant. Each value shown represents the mean \pm the standard error of the mean.

CHAPTER IV

RESULTS

Animal Model

Adult male Sprague Dawley rats were castrated and allowed to recover about two weeks. Each experiment was carried out 15-17 days post castration. At this time, ethanol was administered by an intraperitoneal (i.p.) injection of a 25% v/v (1 cc/80 kg/body weight). Control animals were injected with saline (1cc/80 kg/body weight). Injections for all studies discussed were given between 8:30 A.M. to 9:00 A.M. to control for variations due to daily cycling of hormones.

Figure 2 shows typical blood ethanol concentrations of the ethanol-treated animals. Each value represents the mean \pm standard error of mean (SEM) of 3-6 rats. The timepoints shown represent the number of hours after injection. Blood ethanol concentrations were also determined for control animals (data not shown); however, the levels were too low to measure. These studies showed that ethanol levels decrease from 91 mM at 0.5 hours to 47 mM at 6.0 hours with no measurable ethanol at 24 hours after injection detected. The studies that follow focus on the time points of 1.5 and 3.0 hours after injection (66 mM at 1.5 hrs. and 58 mM at 3.0 hrs.).

The Effect of Ethanol on Luteinizing Hormone Protein and mRNA Levels

Previous studies in the laboratory showed that ethanol affects luteinizing hormone (Emanuele et al. 1991). The studies examined in this dissertation build upon these previous findings; therefore, the same studies were repeated. Results are found in Figures 3-6.

Studies examining the effect of ethanol on serum LH levels supported the results published previously (Cicero et al. 1978; Pohl et al. 1987; Salonen and Huhtaniemi 1990; Emanuele et al. 1991). Figure 3 shows the levels of LH, as assessed by RIA after i.p. ethanol injection. Each value represents the mean \pm SEM of six animals. Serum LH levels 1.5 hours after ethanol injection were significantly lower than LH levels in the control animals ($p < 0.05$) (1520 ± 105 pg/ml for control animals vs. 850 ± 53 pg/ml for ethanol-treated animals). Serum LH values were still significantly suppressed 3 hours after injection ($p < 0.001$) (1650 ± 145 pg/ml for control animals vs. 1035 ± 72 pg/ml for ethanol-treated animals). There was no significant difference between control and ethanol animals at 24 hours ($p > 0.05$) (1730 ± 180 pg/ml for control animals vs. 1270 ± 115 pg/ml for ethanol-treated animals).

Pituitary LH content after ethanol exposure was also assessed by RIA (Figure 4). Each value represents the mean \pm SEM of 6 animals. Pituitary LH levels were significantly higher compared to control animals ($p < 0.05$) at 1.5 hours (1000 ± 90 pg/ml for control animals vs. 2430 ± 300 pg/ml for ethanol-treated animals). No statistically significant differences were found at 3.0 (1240 ± 62

pg/ml for control animals vs. 1730 ± 145 pg/ml for ethanol-treated) or 24 hours (1200 ± 53 pg/ml for control animals vs. 1800 ± 185 pg/ml for ethanol-treated animals).

The mRNA levels for β -LH and the common α -subunit were measured for control and ethanol-treated animals. The steady-state levels were analyzed using cDNA probes on Northern blots (see Materials and Methods). Results of a typical Northern blot probed with β -LH and α -subunit are shown in Figures 5A and 5B, respectively. β -LH mRNA was significantly decreased in the ethanol-treated animals compared to controls at 1.5 hours ($p < 0.05$) (250 ± 22 A.D.U. for control animals vs. 148 ± 37 A.D.U. for ethanol-treated animals). The decrease in β -LH mRNA was greater at 3.0 hours ($p < 0.001$) (320 ± 15 A.D.U. for control animals vs. 53 ± 11 A.D.U. for ethanol-treated animals). By 24 hours, no significant difference was found between control and ethanol-treated animals (315 ± 30 A.D.U. for control animals vs. 287 ± 23 A.D.U. for ethanol-treated animals). Furthermore, the common α -subunit mRNA level was not significantly altered by ethanol treatment (303 ± 107 A.D.U. for control animals vs. 181 ± 43 A.D.U.) for ethanol-treated animals at 1.5 hrs.; 205 ± 25 A.D.U. for control animals vs. 242 ± 32 A.D.U. for ethanol-treated animals at 3.0 hrs.; 267 ± 68 A.D.U. for control animals vs. 220 ± 41 A.D.U. for ethanol-treated animals at 24 hrs.). The Northern blot for 3.0 hours is shown in figure 5B. The same blot from 5A was stripped and reprobed. The results of densitometric analyses, corrected for loading, are shown in Figures 6 A and B. Each value represents the mean and SEM for three animals.

The Effect of Ethanol on Follicle-Stimulating Hormone Protein and mRNA Levels

The effect of ethanol on serum FSH levels was determined by RIA. The experiments were completed following the same protocol used for the LH studies (see above). Serum FSH levels were significantly decreased at 1.5 and 3.0 hours after injection ($p < 0.05$) (59 ± 3 ng/ml for control animals vs. 36 ± 6 ng/ml for ethanol-treated animals at 1.5 hrs.; 57 ± 3 ng/ml for control animals vs. 37 ± 6 ng/ml for ethanol-treated animals at 3.0 hrs.) (Figure 7). However, no measurable suppression was observed 24 hours after injection (61 ± 4 ng/ml for control animals vs. 58 ± 5 ng/ml for ethanol-treated animals). The values represent the mean \pm SEM for five animals.

Ethanol treatment did not affect pituitary FSH content (Figure 8). There was no statistical difference in FSH content at any time point studied (22 ± 5 ng/ml for control animals vs. 18 ± 4 ng/ml for ethanol-treated animals at 1.5 hrs.; 12 ± 2 ng/ml for control animals vs. 14 ± 3 ng/ml for ethanol-treated animals at 3.0 hrs.; 15 ± 1 ng/ml for control animals vs. 19 ± 2 ng/ml for ethanol-treated animals at 24.0 hrs). The FSH levels were assessed by RIA (see Material and Methods). Each value represents the mean \pm SEM for five animals.

Results from Northern blot analysis showed ethanol did not alter steady-state mRNA levels for β -FSH (Figure 9A and B). Figure 8A shows a Northern probed with β -FSH cDNA. There was no

significant difference found between control and ethanol-treated animals at any time point (53 ± 7 A.D.U. for control animals vs. 45 ± 13 A.D.U. for ethanol-treated animals at 1.5 hrs.; 47 ± 11 A.D.U. for control animals vs. 41 ± 6 A.D.U. for ethanol-treated animals at 3.0 hrs.; 62 ± 9 A.D.U. for control animals vs. 54 ± 15 A.D.U. for ethanol-treated animals at 24.0 hrs.). In Figure 9B, the densitometric analysis of the Northern blot was corrected for loading as described in Material and Methods.

Assessment of Modifications of the LH Protein After Ethanol Exposure

LH is a glycoprotein with one oligosaccharide attached to the α -subunit and two oligosaccharides attached to the β -subunit. Ethanol has been shown to effect glycosylation of proteins (Ghosh et al. 1991). Ethanol may be affecting the type of glycosylation of the LH protein. If modifications occurred, the LH protein would be larger or smaller. This size difference could be detected by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis (see Materials and Methods). The studies that follow attempt to detect modification of the LH protein after ethanol exposure by using the forementioned method. These studies used pituitaries from animals that had been injected with ethanol or saline 1.5 hours previously. This time point was chosen based on results above showing a statistically significant increase in LH pituitary content at this time point.

Initial studies were performed using an NIH polyclonal LH antibody. There were several problems using this antibody for analysis of possible size modifications of LH protein. First, the antibody does not distinguish between the α and β subunits. The β -LH subunit should be at 19 Kd and α -subunit at 17 Kd. However, with the above antibody, interaction with the protein is seen as a smear which migrated to 18 Kd (Figure 10A), making it difficult to determine if a shift in either of the LH subunits had occurred. Secondly, the antibody cross reacts with albumin at 66 Kd (Kelley et al. 1990). For these reasons a polyclonal antibody was made using the pGEX overexpression system (see Materials and Methods). Purified LH-pGEX antibody was used on Western blots giving much better resolution of the subunits. Western blot analysis for six pituitaries of control and ethanol treated animals were performed. Figure 10B shows a typical blot. These results show no shift in either of the subunits.

Assessment of the LH Protein in the Golgi Apparatus

Subcellular fractionation was performed on pooled pituitary samples (5 control and 5 ethanol-treated animals). The Golgi fraction was identified by enzyme analysis and electron microscopy. Galactosyltransferase activity was measured for each of the fractions. The only fraction with measurable activity (74 $\mu\text{mol/h} \times \text{mg protein} \times 10^{-6}$) was then analyzed by electron microscopy. The electron microscopy was done by Dr. John McNulty (Loyola University, Chicago, IL). The samples were prepared as described in Materials

and Methods. Pictures were taken showing the vesicles and the tubules identifying the Golgi fraction (Figure 11A). This was done for control and ethanol treated animals. The Golgi fractions were then subjected to SDS-PAGE analysis followed by Western blot analysis (Figure 11B). Quantitation of the amount of LH protein present was done by densitometer scanning. Correction for loading was carried out by scanning major protein bands on Coomassie blue stained gels of control and ethanol-treated lanes. There was no difference in the amount of β -LH protein between control and ethanol samples.

The Effect of Ethanol on LHRH Protein and mRNA

To determine if ethanol was acting at the hypothalamus, affecting LHRH mRNA and protein content, the hypothalami from the rats used in the pituitary studies were removed and used in the following studies. Hypothalami were homogenized in GIT and an aliquot was removed for RIA to determine hypothalamic LHRH content. The remaining homogenate was used for RNA isolation. There was no statistically significant difference in the hypothalamic LHRH content of control and ethanol-treated animals at any of the time points examined (Figure 12). Each value represents the mean \pm SEM of five animals.

Over the past several years, many methods have been used in our laboratory to attempt to quantitate LHRH mRNA. In initial experiments, 5 to 10 hypothalami were pooled together for Northern or slot blot analysis. Using these methods, only a very low signal was detectable. RNase protection assays also resulted in a low signal for

detection of LHRH mRNA from a single hypothalamus, but a reasonable signal was detected with RNA isolated from a LHRH expressing cell line GT-1(Wetsel et al. 1991) (unpublished data). Recently a comparative Reverse Transcription-Polymerase Chain Reaction assay (RT-PCR) has been developed in our laboratory (Kelley et al. 1993). In this method histone H3.3 is used as an internal control for both the reverse transcription and the thermal amplification steps. H3.3 is a cell-cycle independent gene and constitutively expressed in all tissues (Sittman et al. 1981; Wells et al. 1987). Optimal conditions for this assay were established for the combined use of oligonucleotides for H3.3 and LHRH. The range of linearity with regard to number of amplification cycles was determined to be between 20-25 cycles (Figure 13). This figure shows the effect of increasing the number of amplifications on the amounts of H3.3 and LHRH product generated. Since both H3.3 and LHRH reach a plateau around 25 cycles (Figure 13 B and C) and the range of linearity was between 20 and 25, 22 cycles was chosen for the rest of the experiments. The effect of increasing RNA concentrations in the reverse transcription reaction on the LHRH and H3.3 products are shown in Figure 14. There is a linear increase in LHRH and H3.3 signal with increasing amounts of RNA up to 4 μ g (LHRH, $r=.972$, $p=.020$; H3.3, $r=.980$, $p=.028$).

Since H3.3 was used as an internal control, it was important to demonstrate that H3.3 was not affected by ethanol. Figure 15A shows the H3.3 products for control (lanes 1-5) and ethanol (lanes 6-10) treated samples. Figure 15 B shows the densitometric analysis of

the blot shown in Figure 15 A. The above was repeated twice with both trials showing similarly that H3.3 was not affected by ethanol exposure.

Once the above conditions were established, RT-PCR assay was used to determine the effect of ethanol on LHRH mRNA. A typical autoradiogram of the PCR products is shown in Figure 16 A. Figure 16 B is the densitometric scanning of autoradiograms. It shows no significant difference between control and ethanol-treated animal LHRH mRNA (44 ± 9 A.D.U. for control animals vs. 51 ± 9 A.D.U. for ethanol-treated animals at 1.5 hrs.; 54 ± 13 A.D.U. for control animals vs. 47 ± 8 for ethanol-treated animals at 3.0 hrs.; 61 ± 16 for control animals vs. 86 ± 11 for ethanol-treated animals at 24.0 hrs.). Each value represents the mean \pm SEM of 4-6 animals.

Polysome Profile Analysis of Gonadotropin mRNA After Ethanol Exposure

To further explore the impact of EtOH on the gonadotropins, the pattern of association of gonadotropin mRNA with polysomes was assessed. Cytoplasmic extracts of anterior pituitary glands from control and ethanol-treated animals (3.0 hours after injection) were fractionated through 10 to 40% (wt/vol) sucrose gradients. The gradient was divided into 10 fractions, RNA was extracted from each gradient fraction and subjected to Northern blot analysis.

Results from Northern blots probed with β -LH cDNA show a shift from the heavy polysome fractions (9 and 10) to the lighter polysome fractions (6-8) for ethanol treated animals (Figure 17 A

and B) compared to control animals. This localization of β -LH mRNA to polysome fractions was due to specific ribosomal association since transcripts were released from polysomes in the presence of EDTA (Figure 18). In order to compare the fractions of control and ethanol treated samples, the absorbance at 254 nm was taken of each fraction to determine which of the fractions being compared were from the same fraction of the gradient (Figure 19 A). Figure 19 B and C show the distribution of β -LH mRNA in each fraction in percent of total β -LH mRNA. Figure 20 shows the distribution of dissociated polysomes (treated with EDTA) for control animals (Figure 20B) and ethanol-treated animals (Figure 20C).

The Northern blots for the intact polysomes and dissociated polysomes (data not shown) were stripped and reprobed with α -subunit cDNA and β -FSH cDNA. The polysome distribution of α -subunit mRNA is shown in Figure 21. Panel A and B are the Northern blots for control and ethanol-treated animals, respectively. The densitometric scan of the blots are presented below (Figure 21 C and D). There was no shift in association of the α -subunit mRNA with polysomes when comparing the ethanol-treated and control animals. Similar results were seen when the blots were probed with β -FSH (Figure 22 A-D).

To determine if the shift in β -LH mRNA association with polysomes was due to the ethanol exposure at 3.0 hours post injection and not inherent to the β -LH mRNA, polysome distribution analysis of β -LH mRNA was assessed 24 hours after ethanol injection (Figure 23 A-D). The results showed no change in the association of

β -LH mRNA with polysomes between control and ethanol treated samples.

Assessment of LH Heteronuclear RNA (hnRNA) After Ethanol Exposure

S1 nuclease protection assays were performed to quantitate the levels of hnRNA for β -LH. Oligonucleotide for β -LH was designed to be complementary to 18 nucleotides of β -LH exon 1, 18 nucleotides of β -LH intron and have 6 nucleotides on the 3' end that were not complementary. The oligonucleotides were designed to distinguish between undigested probe and the RNA:DNA hybrids created from hybridization and S1 nuclease treatment of pituitary RNA and probe. The product representing LH hnRNA, the RNA:DNA hybrids formed, would be 36 nucleotides long and distinct from the 42 nucleotide undigested probe when electrophoresed on a 15% / 8M Urea polyacrylamide gel. A second oligonucleotide was designed to be complementary to H3.3. It also had 6 non-complementary nucleotides added to its 3' end. The undigested probe was 27 nucleotides long, while the product representing the RNA:DNA hybrids were 21 nucleotides long. H3.3 was used in the reaction as a loading control for amount of RNA added to the reaction.

Three pituitaries were pooled together to isolate enough RNA to detect β -LH hnRNA. Pituitaries from control and ethanol-treated animals 3 hours post-injection were used for these studies. There was no significant difference detected in the level of β -LH hnRNA when comparing control and ethanol samples (Figure 24). Amount of RNA per reaction was corrected for with H3.3 levels detected on the

gel. The products at 42 and 27 represent undigested LH and H3.3 probes, respectively. The H3.3 RNA:DNA product run at 21 and the β LH hnRNA RNA:DNA product run at 36. In Figure 24 A, lane 1 is untreated probe, lane 2 is undigested probe alone(no RNA was added to the reaction). Lanes 3, 5, and 7 are reactions with control sample, and lanes 4,6, and 8 are reactions with ethanol-treated samples. Each value in Figure 24 B represents the mean \pm SEM for three reactions (each reaction contained RNA from three pituitaries).

Figure 2. Blood Ethanol Concentrations of Animals After Time of Injection. Serum ethanol concentrations were determined on trunk blood and determined using an ethanol kit from Sigma (see Materials and Methods). This data represents the mean ethanol concentration \pm SEM of 6-12 animals at each time point.

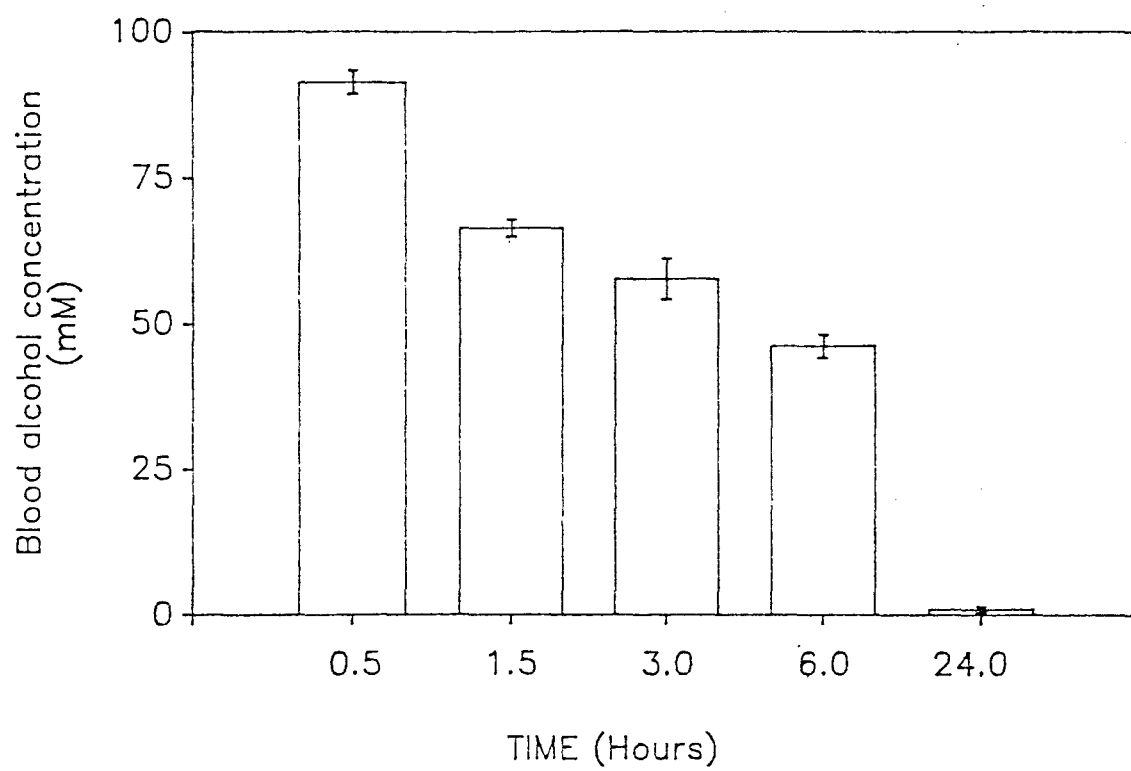


Figure 3. The Effect of EtOH on Serum LH Levels. Serum LH levels were quantitated using RIA (see Materials and Methods). Twelve rats were used at each time point, 6 control and 6 EtOH. The symbols * and ** indicated values from control ethanol-treated animals differ significantly at $p < 0.05$ and $p < 0.001$ respectively. Values are mean serum LH levels \pm SEM.

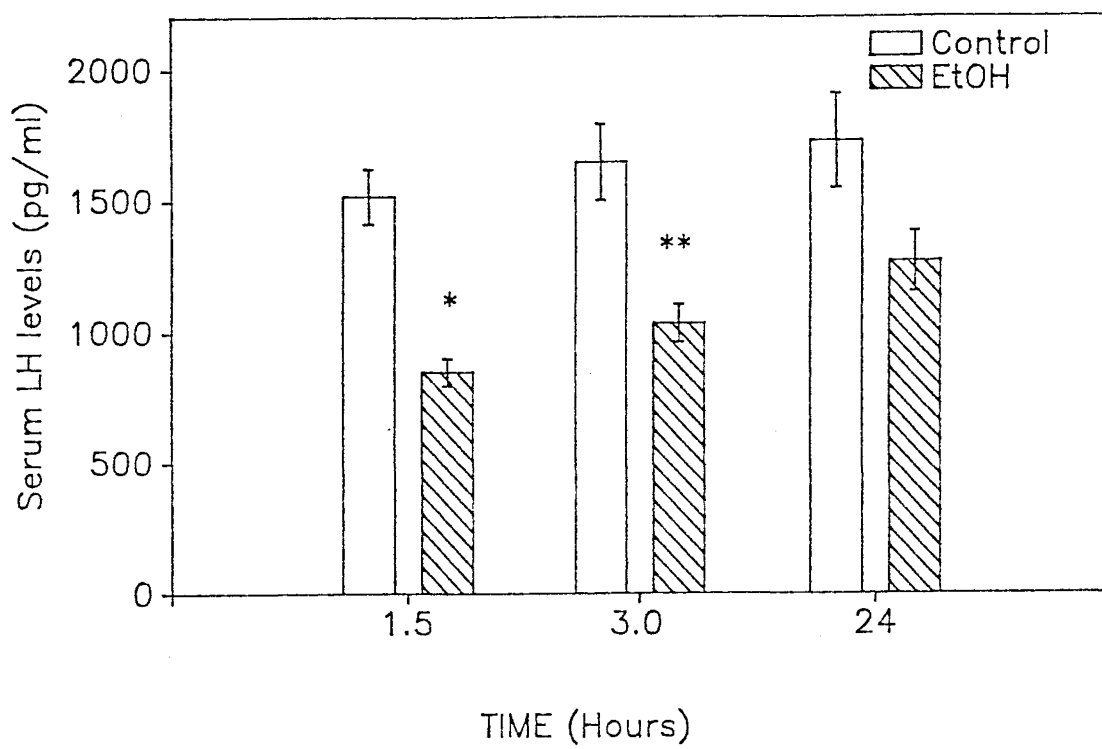


Figure 4. The Effect of Ethanol on the Intrapituitary LH Content. Intrapituitary LH levels were quantitated using RIA (see Materials and Methods). Twelve rats were used at each time point, 6 control and 6 EtOH. Values are the mean LH levels \pm SEM. * $p < 0.05$.

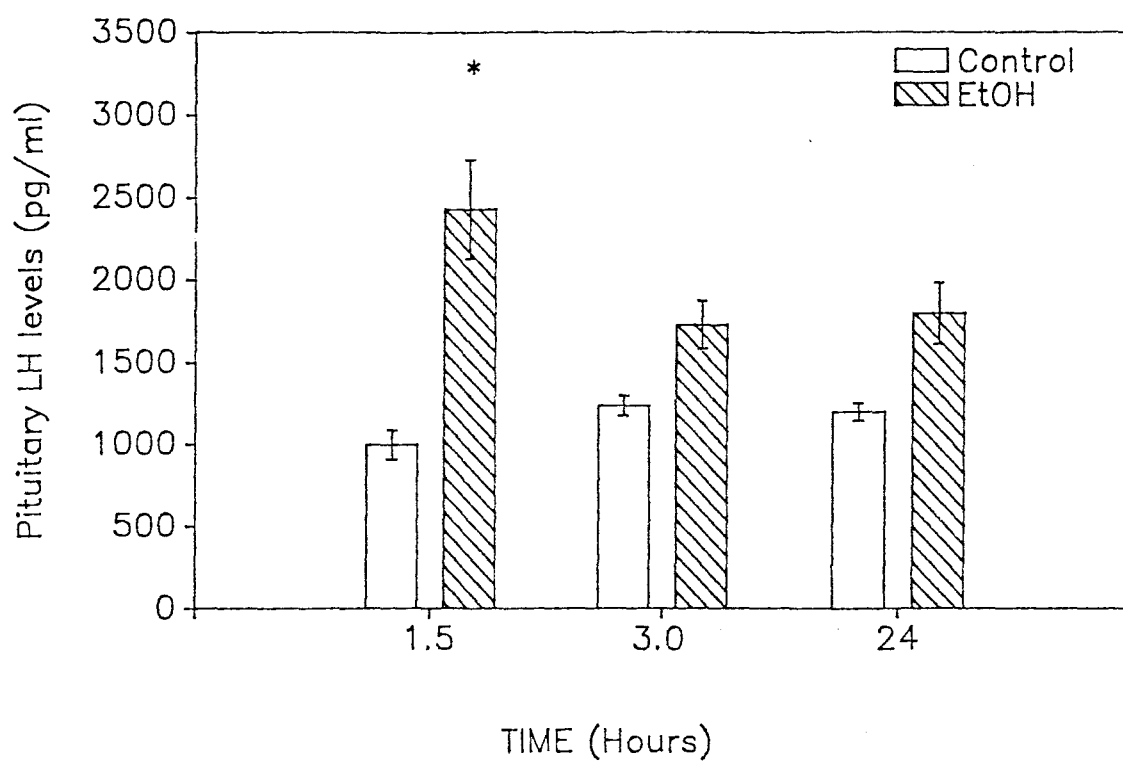


Figure 5. The Effect of Ethanol on β -Subunit of LH mRNA. A Northern blot of total RNA is shown. Lanes 1-3 are control rats and lanes 4-6 are ethanol-injected rats. The times after i.p. injection, in hours are shown on the left. (A) Blot probed with β -LH cDNA. (B) Blot in (A) stripped and reprobed with α -LH subunit cDNA clone.

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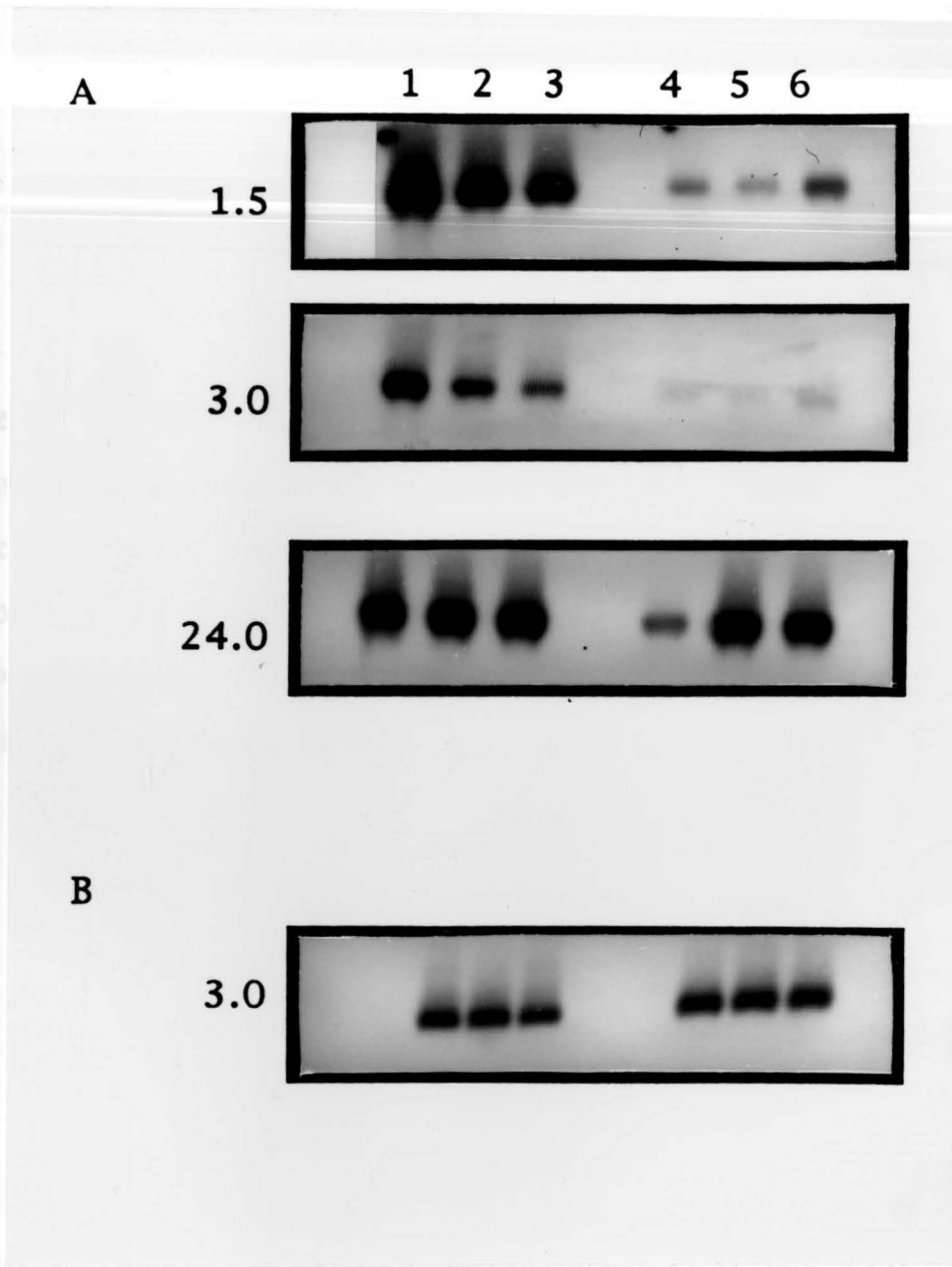


Figure 6. The Effect of Ethanol on α - and β -Subunit of LH mRNA. Densitometric analysis of the Northern blots shown in Figure 4. Data was corrected for loading differences as described in Materials and Methods. Each value represents the mean mRNA in arbitrary densitometer units (A.D.U.) \pm SEM. (A) β -LH subunit mRNA. (B) α -subunit mRNA at each of three time points after injection.

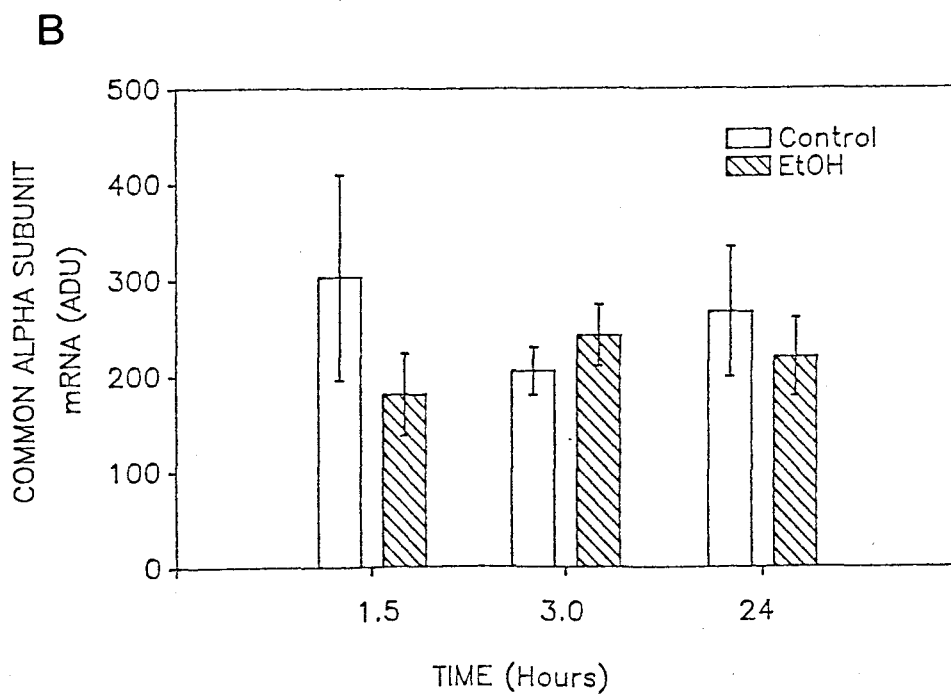
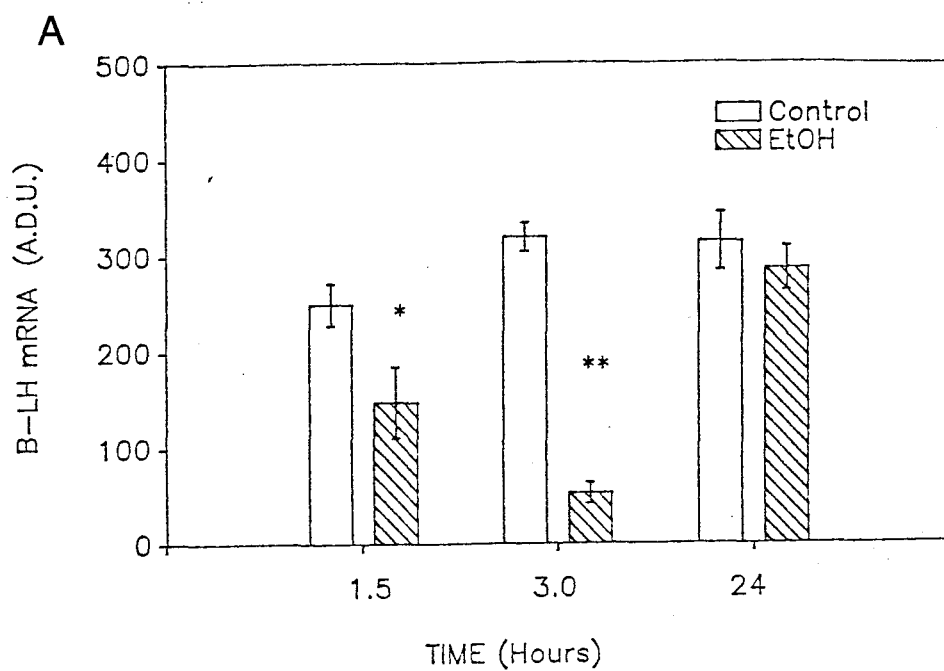


Figure 7. The Effect of Ethanol on Serum FSH Levels. Serum FSH levels were quantitated using RIA (see Materials and Methods). Each value represents the mean of the five animals \pm SEM. The symbol * indicates values from control and ethanol-treated animals differ significantly at $p < 0.05$. There was no significant difference between control and ethanol-treated animals 24 hours after injection.

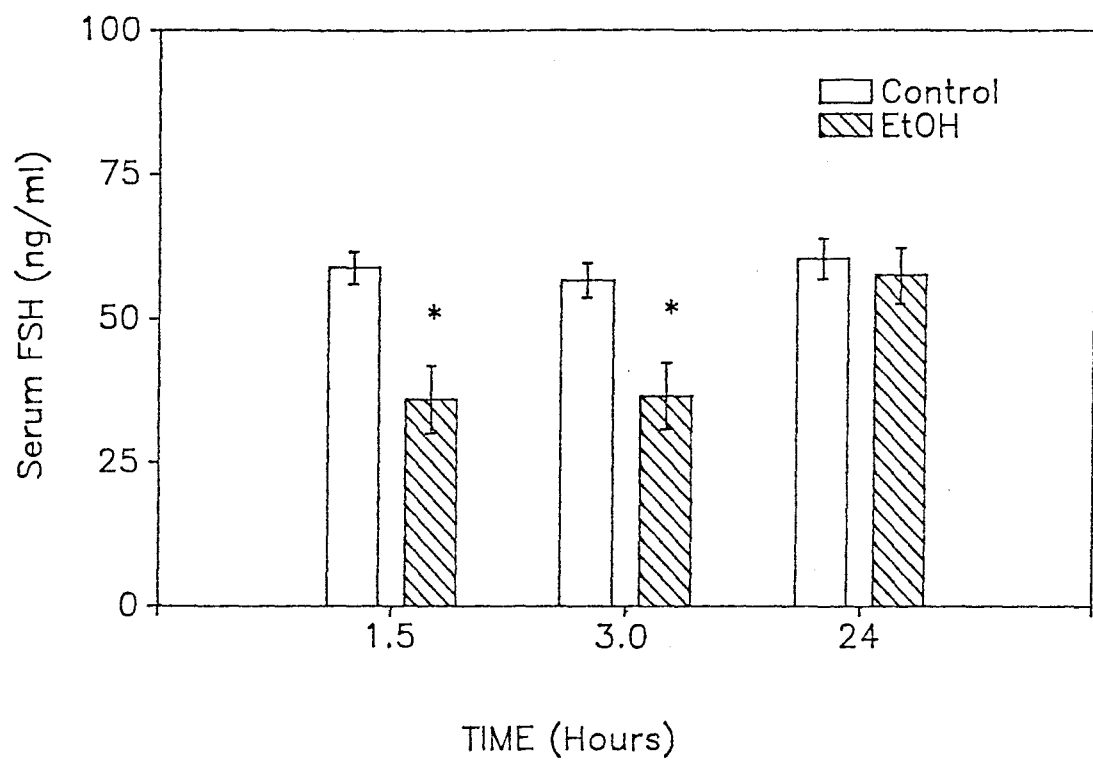


Figure 8. The Effect of Ethanol on the Intrapituitary FSH Content. Intrapituitary FSH levels were quantitated using RIA (see Materials and Methods). Each value represents the mean of FSH level \pm SEM. Ten rats were used at each time point, 5 control and 5 ethanol i.p. injected rats. There was no significant difference found between control and ethanol-treated animals at 1.5 hours, 3.0 hours, or 24 hours after injection.

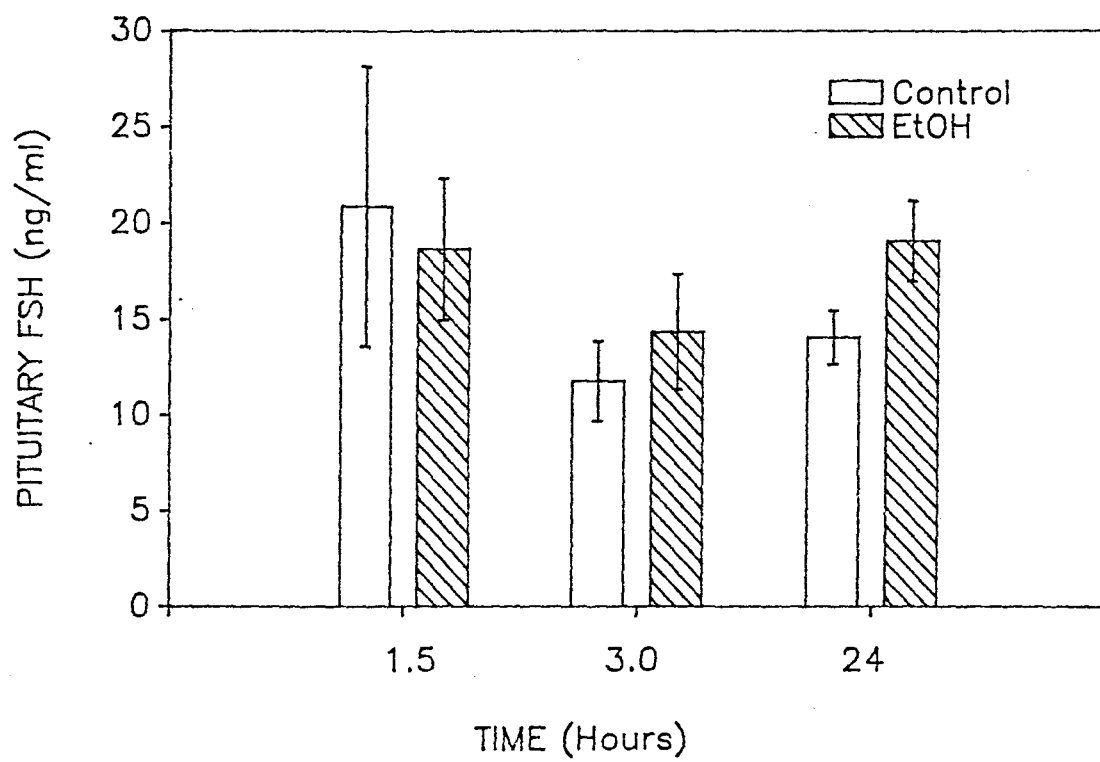
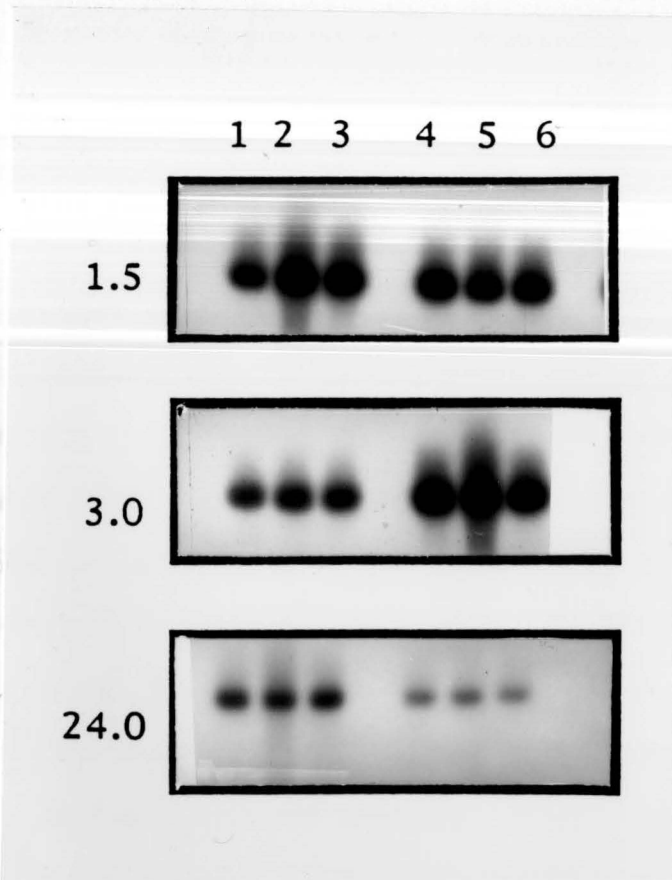


Figure 9. The Effect of Ethanol on β -FSH mRNA. (A) Northern blot analysis of β -FSH mRNA. 1.5, 3.0, and 24 represent the hours after ethanol injection. Lanes 1-3 are control animals. Lanes 4-6 are ethanol-treated animals. (B) Densitometric analysis of Northern blots probed with β -FSH cDNA. Data was corrected for loading differences as described in Materials and Methods. Each bar represents the mean mRNA levels in arbitrary densitometer units (A.D.U.) \pm SEM of 3-6 animals. There was no significant difference found between control and ethanol-treated animals at 1.5 hours, 3.0 hours, or 24 hours after injection.

A



B

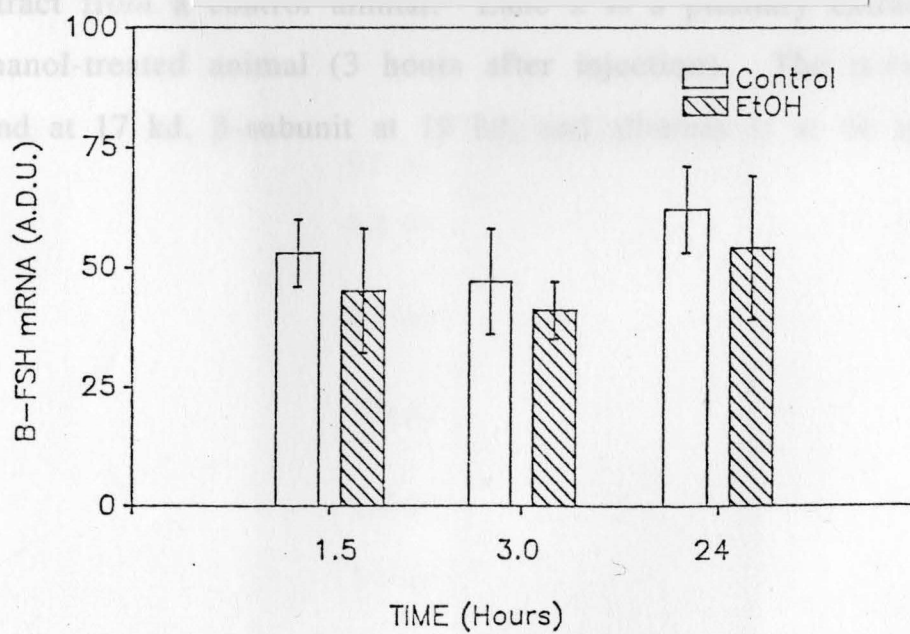


Figure 10. The Effect of Ethanol on LH Protein as Determined by Western Blot Analysis. (A) Equal amounts of protein were loaded, run on 12% SDS-polyacrylamide gel, electroblotted, and incubated with NIH polyclonal LH antibody. Lane 1 is a pituitary extract from a control animal. Lane 2 is a pituitary extract from an ethanol-treated animal (3 hours after injection). (B) Same as (A) except blot was incubated with LHpGex polyclonal antibody. Lane 1 is a pituitary extract from a control animal. Lane 2 is a pituitary extract from an ethanol-treated animal (3 hours after injection). The α -subunit is the band at 17 kd, β -subunit at 19 kd, and albumin is at 66 kd.

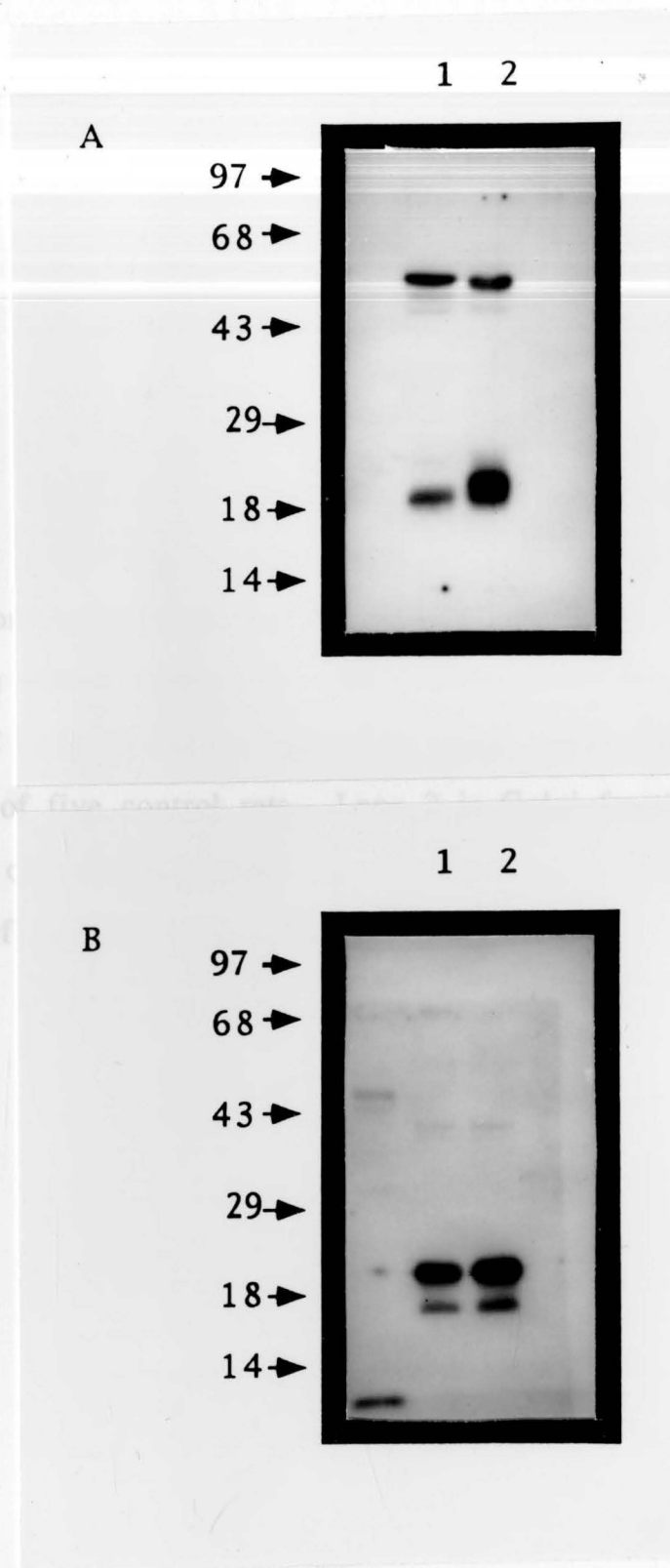
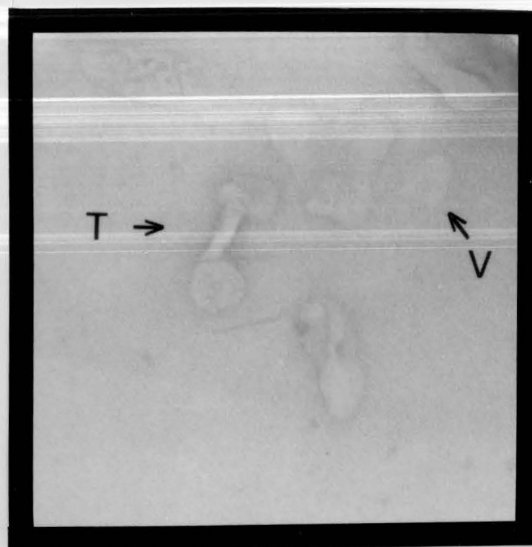


Figure 11. The Effect of Ethanol on LH Protein in the Golgi Apparatus. (A) Electron microscopy of Golgi fraction of subcellular fractionation preparation (see Materials and Methods). The preparation shows tubules (T) and vesicles (V). (B) Western blot analysis of Golgi fraction. Lane 1 is Golgi fraction obtained from pituitaries of five control rats. Lane 2 is Golgi fraction from five pituitaries of ethanol-injected rats (3 hours after injection). Equal amounts of protein were loaded.

A



B

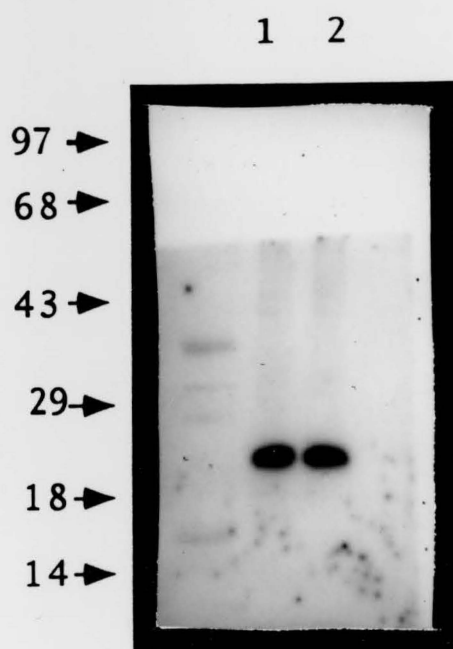


Figure 12. The Effect of Ethanol on Hypothalamic LHRH Content.

LHRH levels were quantitated using RIA. Ten animals were used at each time point, five control and five ethanol. Each value represents the mean LHRH level \pm SEM. There was no significant difference found between control and ethanol-treated animals at 1.5 hours, 3.0 hours, or 24 hours after injection.

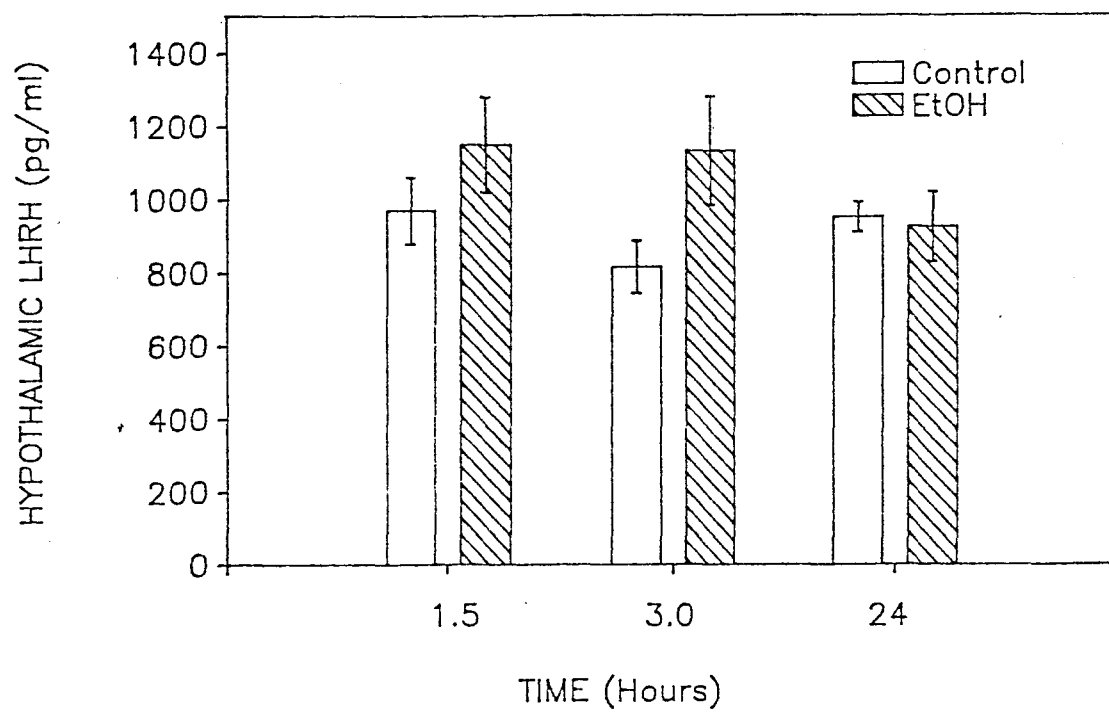


Figure 13. The Effect of Increasing Numbers of Amplification Cycles on LHRH and H3.3 Signals. (A) Total RNA (3 μ g) from hypothalami of castrated male rats were subjected to RT-PCR (see Materials and Methods). Aliquots were removed at 15,20,25,30,35,40 cycles. (B) densitometric scanning of (A). (C) densitometric scanning of a blot in of samples treated as in (A) but aliquots were removed at 20,21,22,23,24,25 cycles and only contained LHRH oligonucleotides.

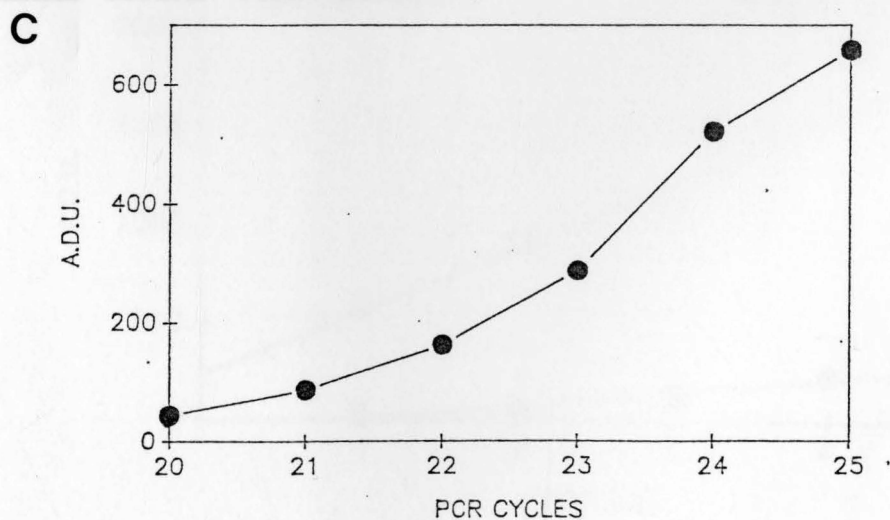
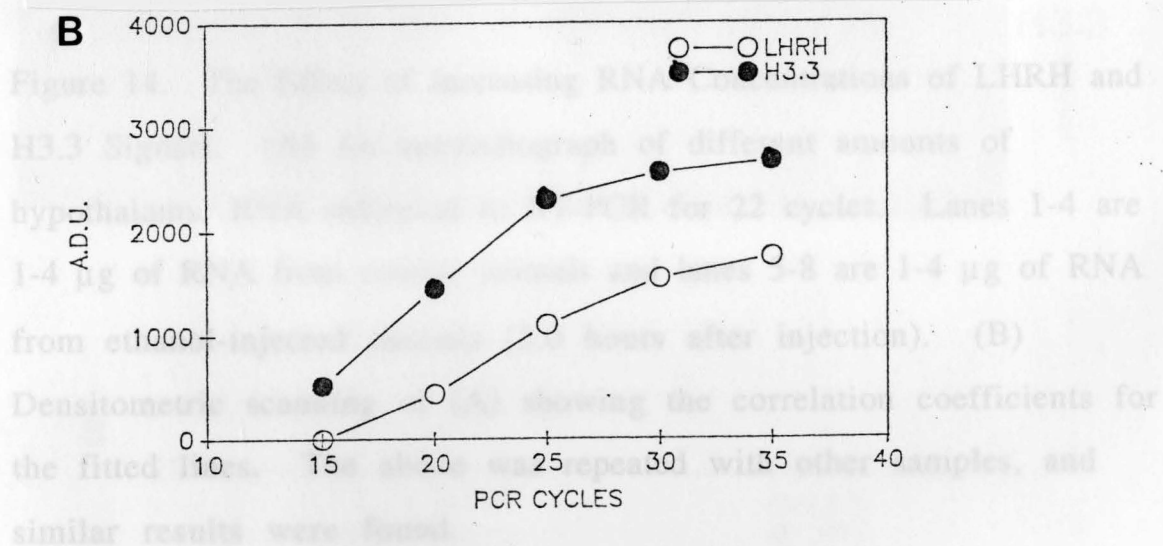
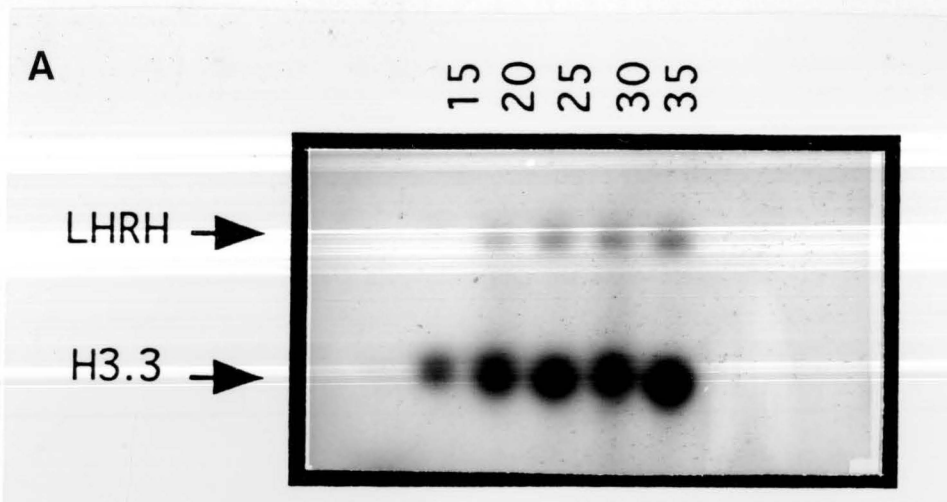
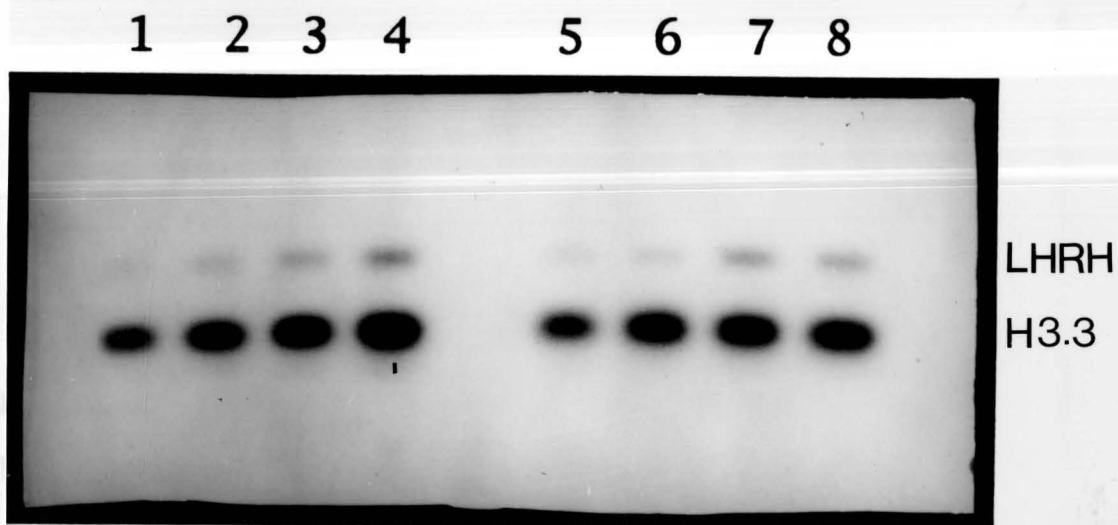


Figure 14. The Effect of Increasing RNA Concentrations of LHRH and H3.3 Signals. (A) An autoradiograph of different amounts of hypothalamic RNA subjected to RT-PCR for 22 cycles. Lanes 1-4 are 1-4 μ g of RNA from control animals and lanes 5-8 are 1-4 μ g of RNA from ethanol-injected animals (3.0 hours after injection). (B) Densitometric scanning of (A) showing the correlation coefficients for the fitted lines. The above was repeated with other samples, and similar results were found.

A



B

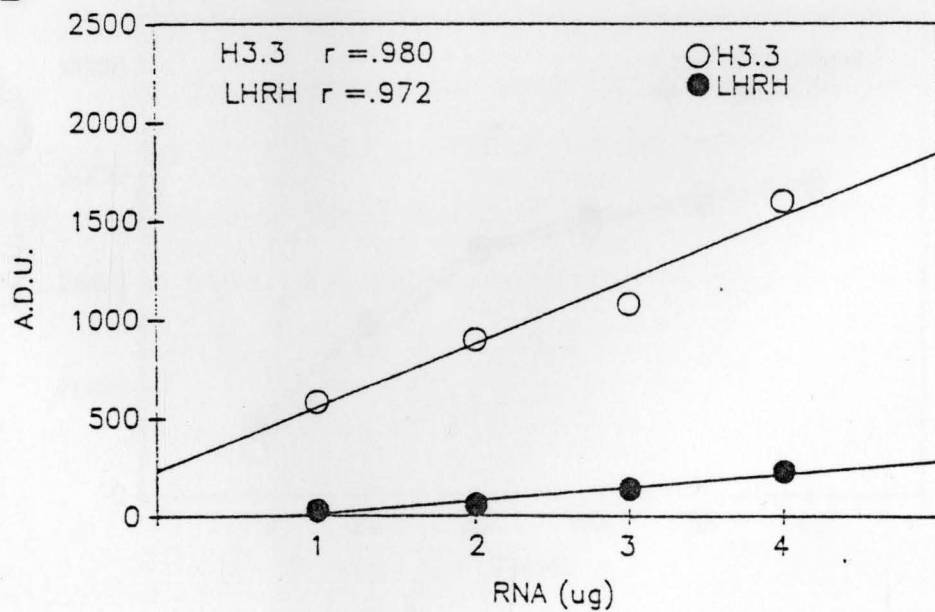


Figure 15. The Effect of Ethanol on H.3.3. (A) RT-PCR blot of 3 μ g of hypothalamic RNA from control animals, left side of panel, and ethanol-injected animals (3 hours after injection), right side of panel. Aliquots were removed after 15,20,25,30,35,40 cycles. (B) Densitometric scanning of (A). The above was repeated and similar results were found.

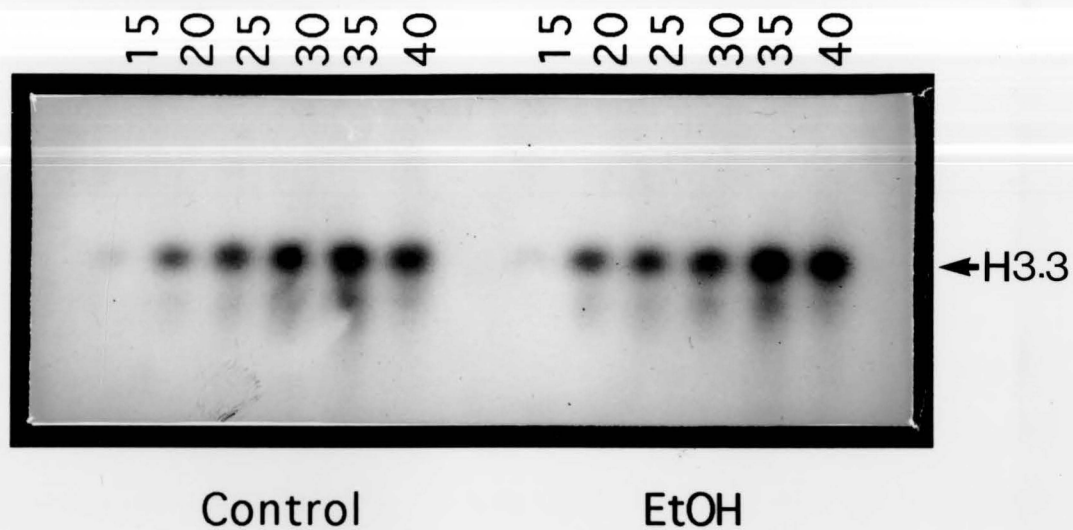
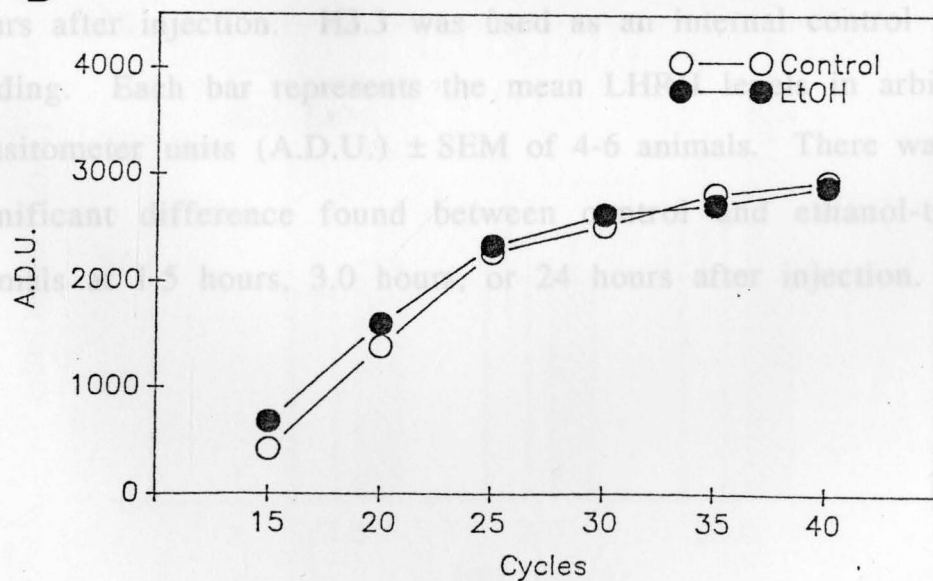
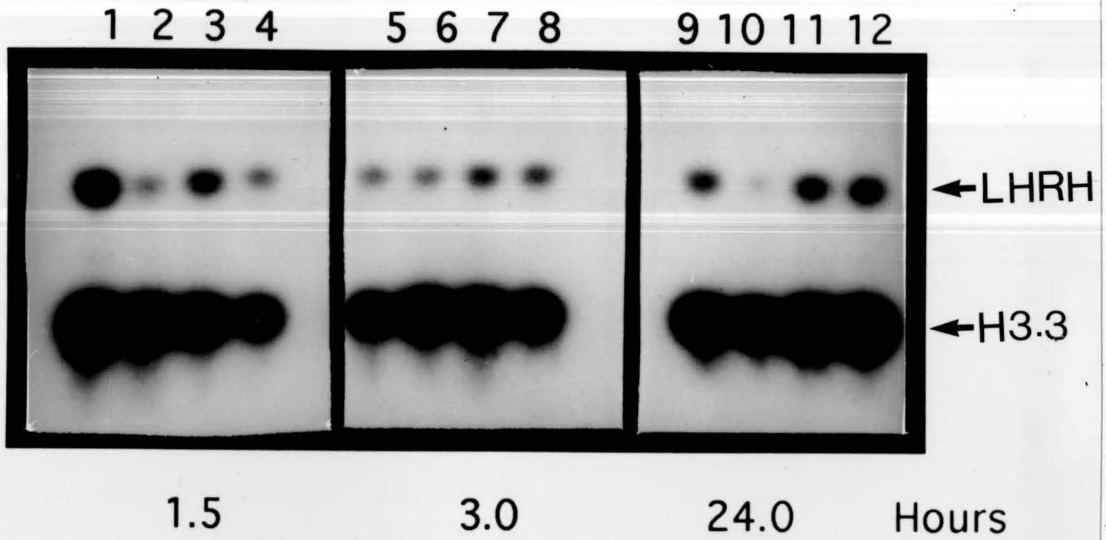
A**B**

Figure 16. The Effects of Ethanol on LHRH mRNA. (A) Typical RT-PCR autoradiogram of samples 1.5, 3.0, and 24.0 hours post injection (see Materials and Methods). Lanes 1, 2, 5, 6, 9, and 10 are samples from control animals; lanes 3, 4, 7, 8, 11, and 12 are from ethanol-injected animals. (B) Densitometric scanning of RT-PCR autoradiograms of control and ethanol-injected animals at 1.5, 3.0, 24 hours after injection. H3.3 was used as an internal control for loading. Each bar represents the mean LHRH levels in arbitrary densitometer units (A.D.U.) \pm SEM of 4-6 animals. There was no significant difference found between control and ethanol-treated animals at 1.5 hours, 3.0 hours, or 24 hours after injection.

A



B

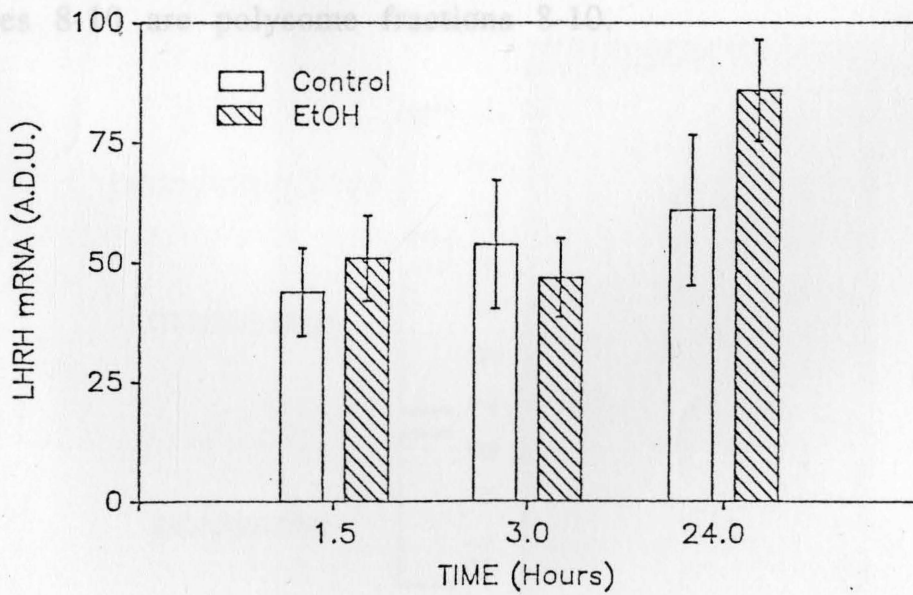


Figure 17. Polysomal Distribution of β -LH mRNA in HKM buffer. Representative Northern blot of sucrose gradient fractions in HKM buffer probed with β -LH (see Materials and Methods) of (a) five pooled pituitaries of control animals, (b) five pooled pituitaries of ethanol-injected animals (3.0 hours after injection). Lanes 1-3 are postpolysomal fractions 1-3. Lanes 4-7 are monosome fractions 4-7. Lanes 8-10 are polysome fractions 8-10.

A

Control



1 2 3 4 5 6 7 8 9 10

postpolysomal

monosome

polysome

B

EtOH



1 2 3 4 5 6 7 8 9 10

postpolysomal

monosome

polysome

Figure 18. Polysomal Distribution of β -LH mRNA in HKE buffer. Representative Northern blot analysis of sucrose gradient fractions in HKE buffer probed with β -LH (see Materials and Methods) of (a) five pooled pituitaries of control animals, (b) five pooled pituitaries of ethanol-injected animals (3.0 hours after injection). Lanes 1-3 are postpolysomal fractions 1-3. Lanes 4-7 are monosome fractions 4-7. Lanes 8-10 are polysome fractions 8-10.

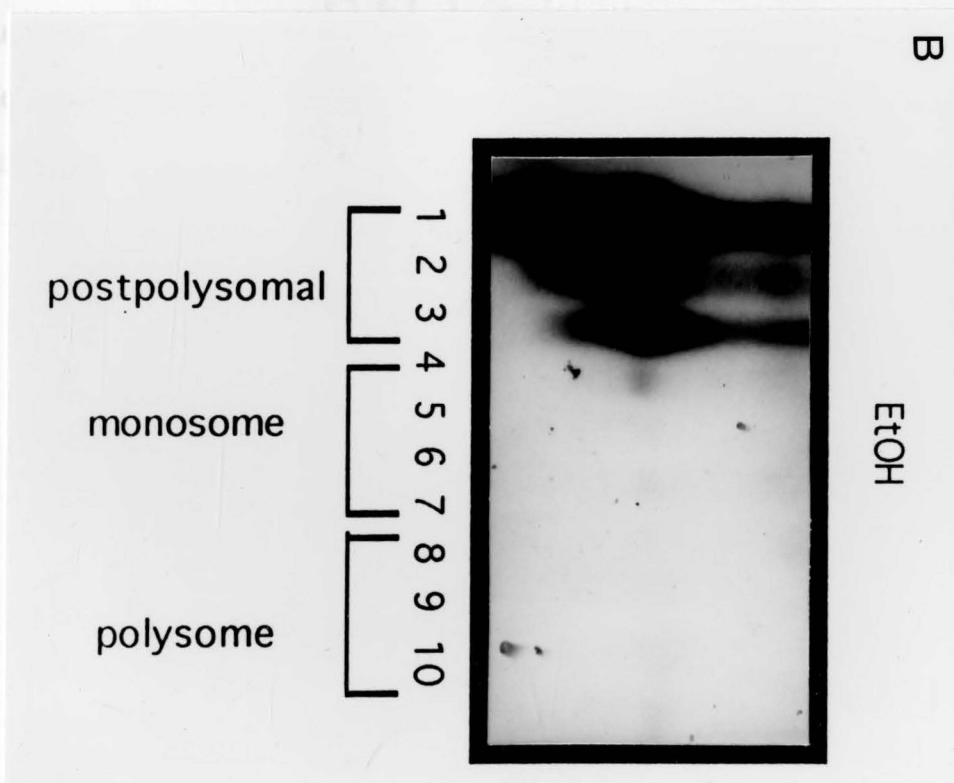
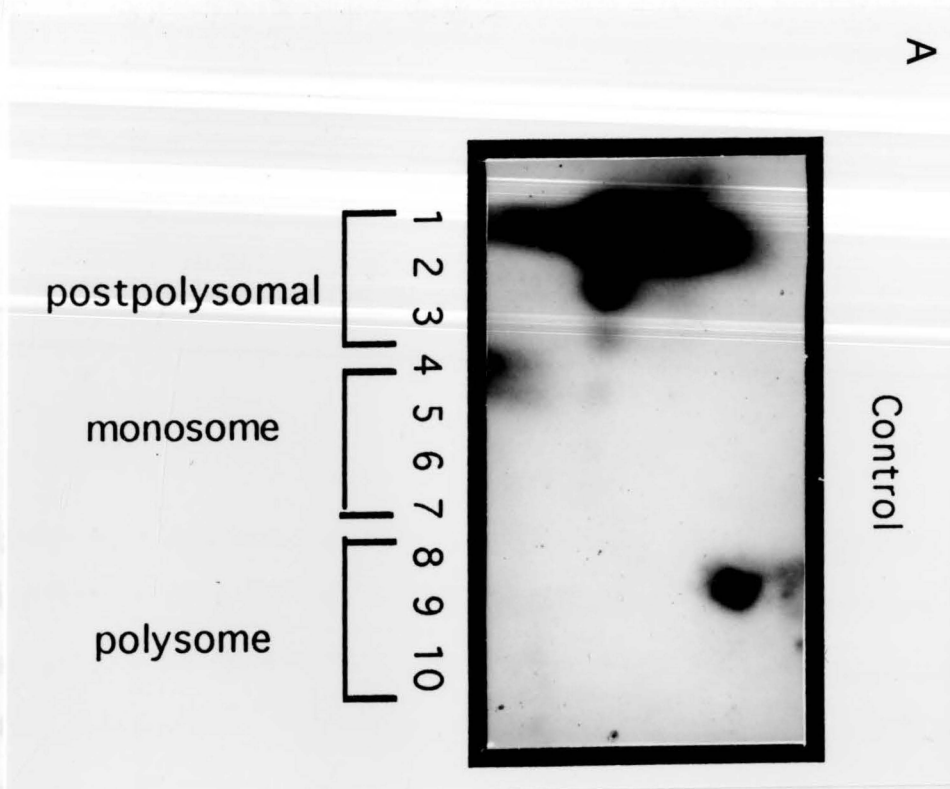


Figure 19. Optical Density Measurements of the Polysomal Distribution of β -LH mRNA in HKM Buffer. (A) Absorbance of fractions 1-10 at 254 nm for control and ethanol-treated samples of intact polysomes. (B) Percent of total β -LH mRNA in each of the fractions with the corresponding optical density (254 nm) for control samples. (C) Percent of total β -LH mRNA in each of the fractions with the corresponding optical density (254 nm) for ethanol-treated samples.

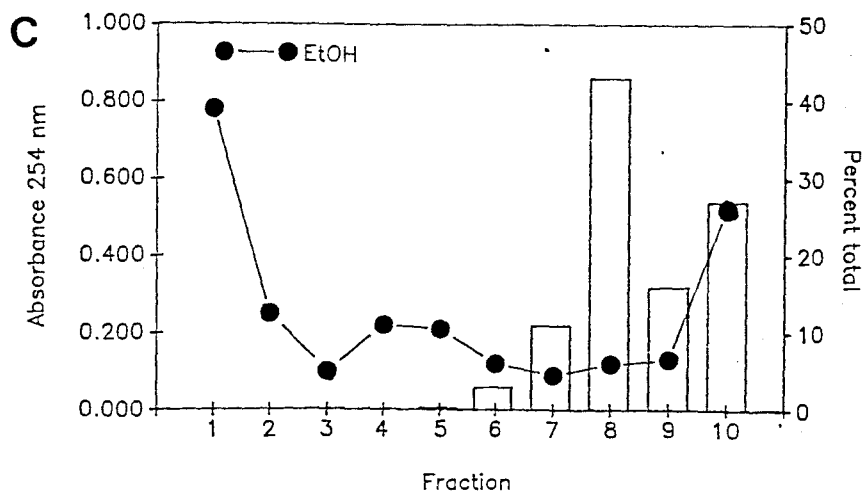
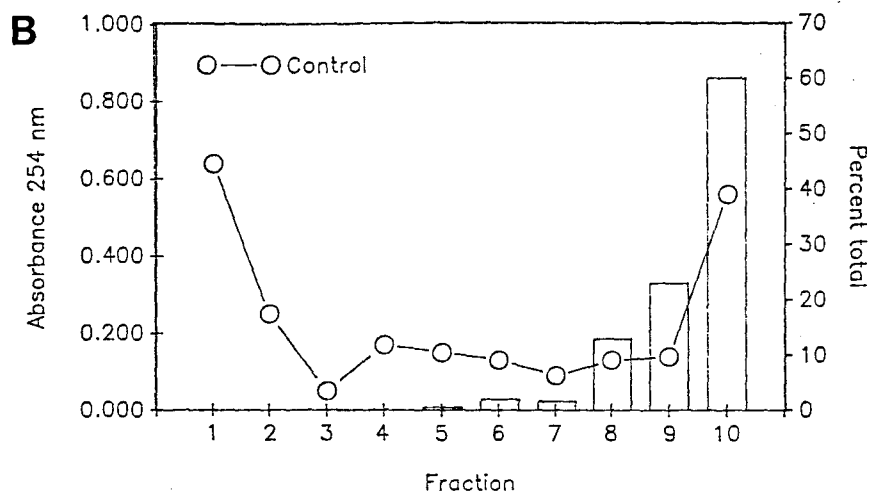
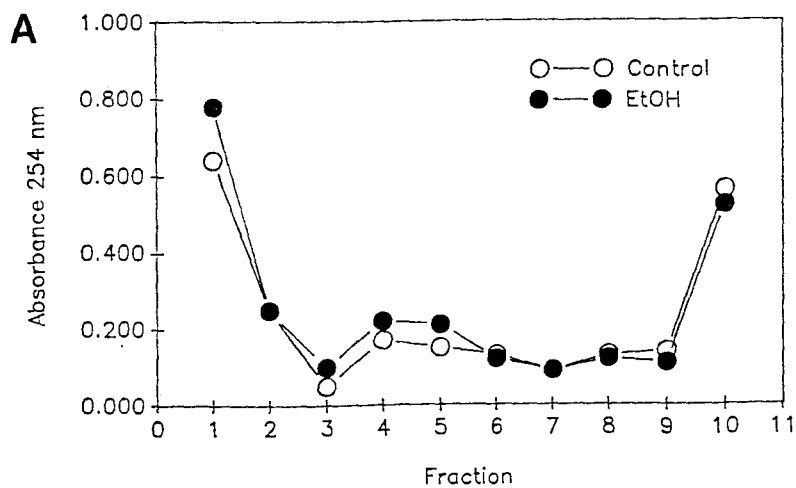


Figure 20. Optical Density Measurements of the Polysomal Distribution of β -LH mRNA in HKE Buffer. (A) Absorbance of fractions 1-10 at 254 nm for control and ethanol-treated samples of disassociated polysomes. (B) Percent of total β -LH mRNA in each of the fractions with the corresponding optical density (254 nm) for control samples. (C) Percent of total β -LH mRNA in each of the fractions with the corresponding optical density (254 nm) for ethanol-treated samples.

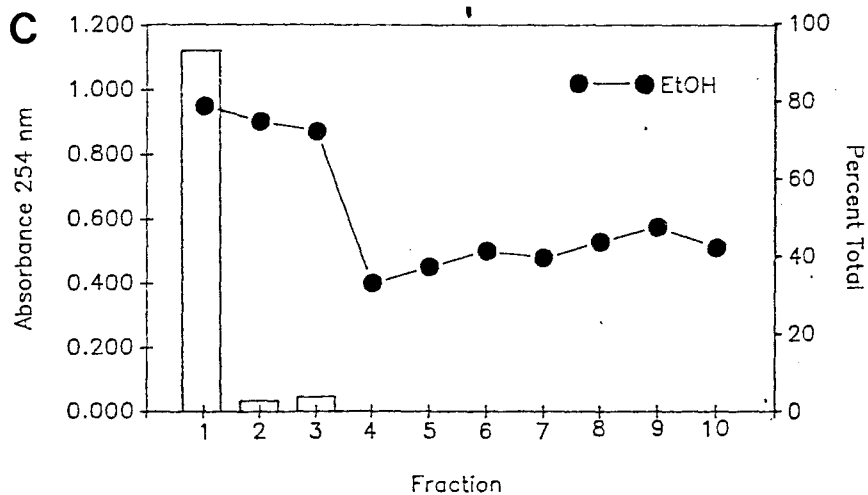
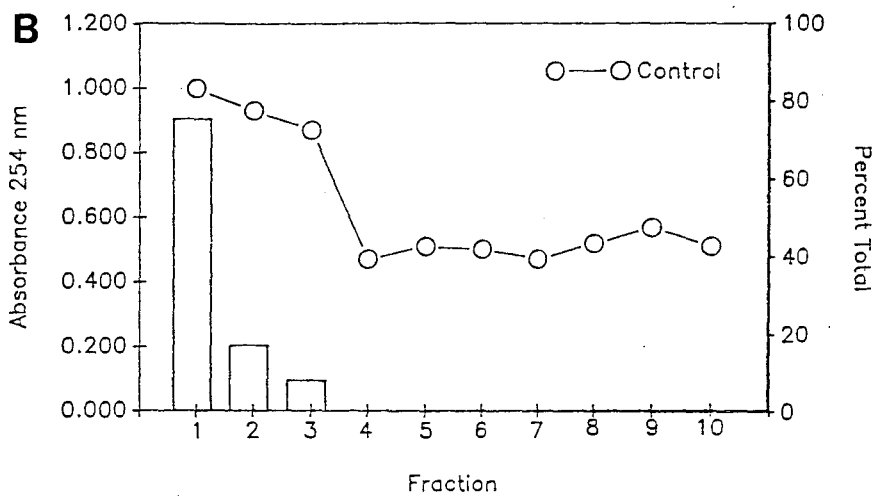
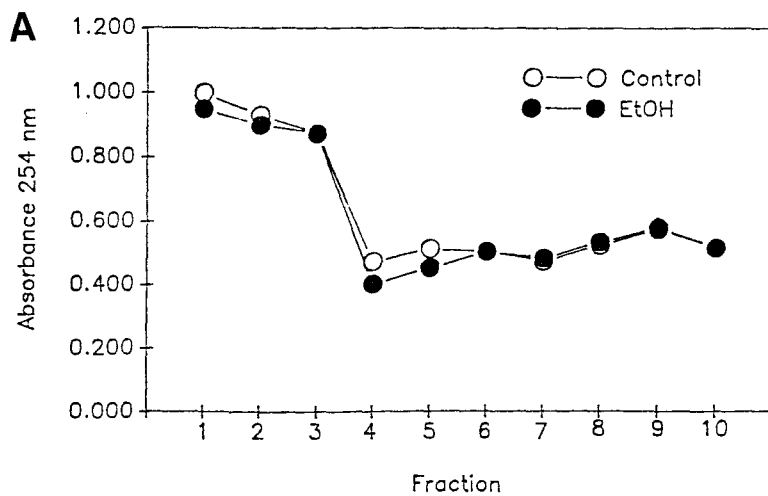


Figure 21. Polysomal Distribution of α -subunit mRNA in HKM Buffer. (A) Northern blot from Figure 16 (a) stripped and reprobed with α -subunit cDNA. (B) Northern blot from Figure 16 (b) stripped and reprobed with α -subunit cDNA. (C) Densitometric scan of (A). (D) Densitometric scan of (B).

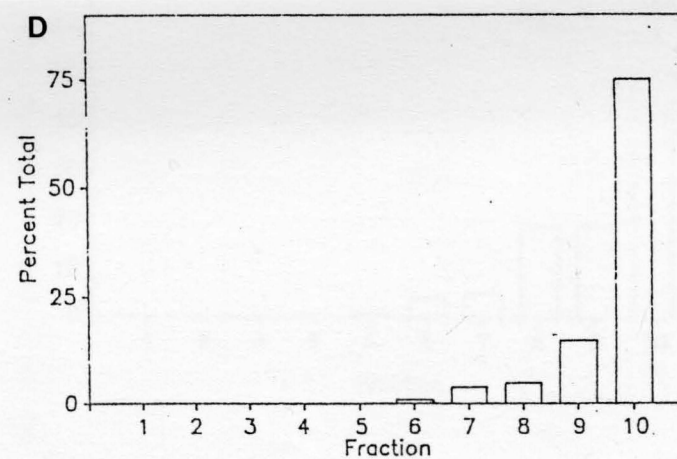
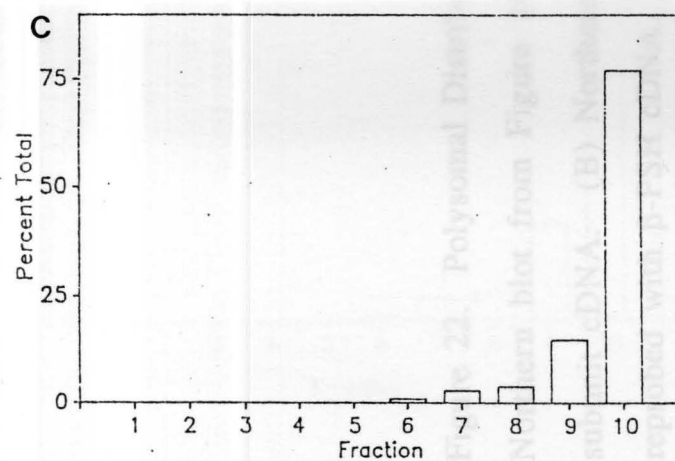
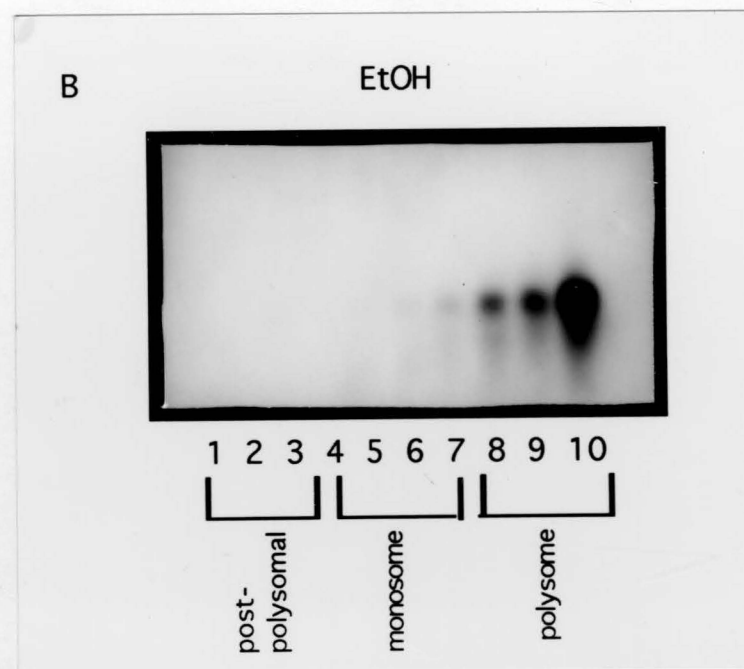
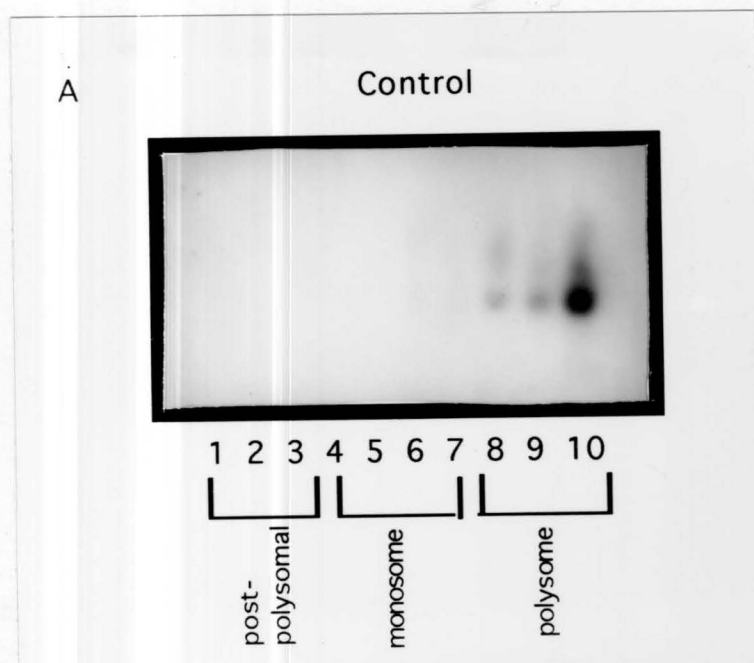


Figure 22. Polysomal Distribution of β -FSH mRNA in HKM Buffer. (A) Northern blot from Figure 16 (a) stripped and reprobbed with β -FSH subunit cDNA. (B) Northern blot from Figure 16 (b) stripped and reprobbed with β -FSH cDNA. (C) Densitometric scan of (A). (D) Densitometric scan of (B).

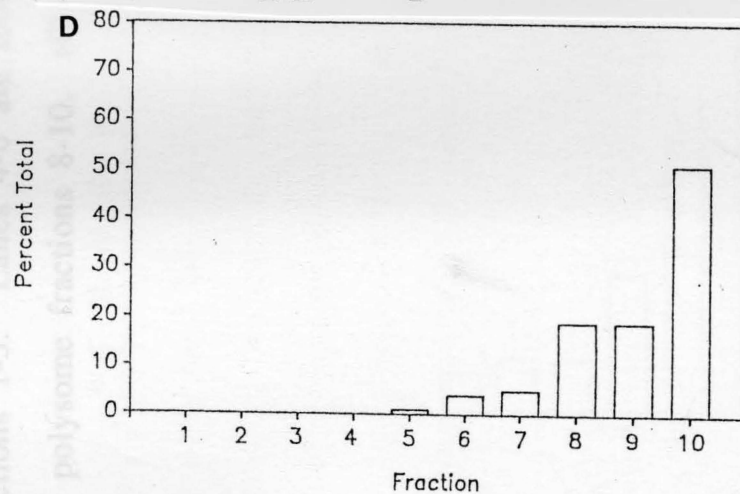
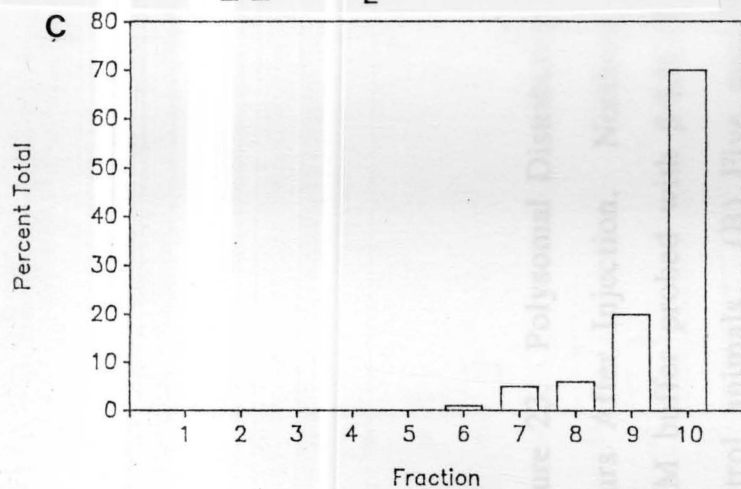
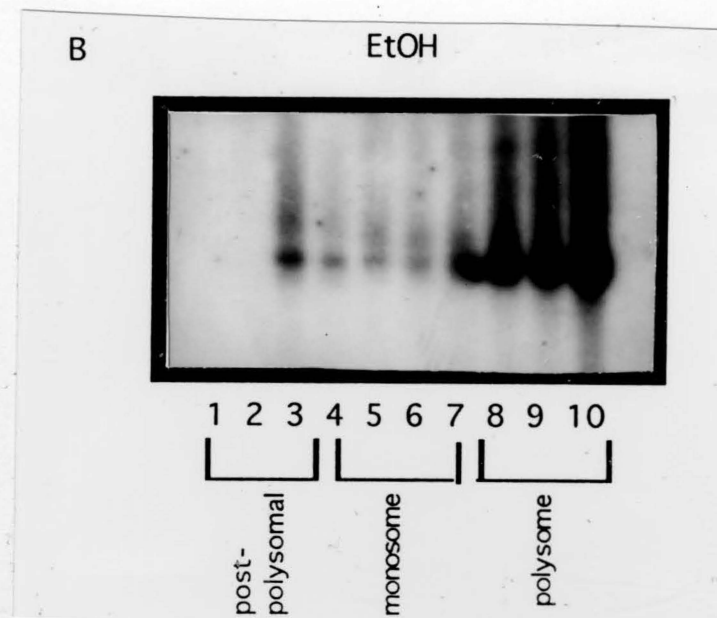
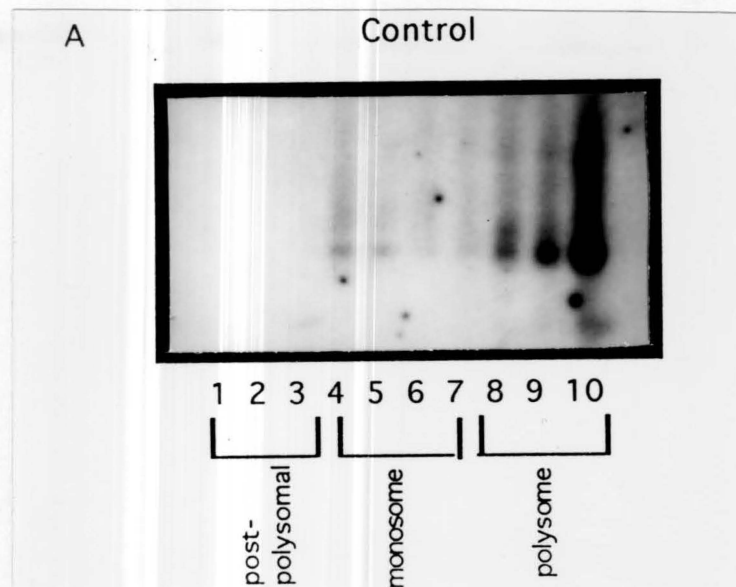


Figure 23. Polysomal Distribution of β -LH mRNA in HKM Buffer 24 Hours After Injection. Northern blot analysis of sucrose gradient in HKM buffer probed with β -LH cDNA. (A) Five pooled pituitaries of control animals. (B) Five pooled pituitaries of ethanol-injected animals (24 hours after injection). Lanes 1-3 are postpolysomal fractions 1-3. Lanes 4-6 are monosome fractions 4-6. Lanes 8-10 are polysome fractions 8-10. (C & D) Densitometric analysis.

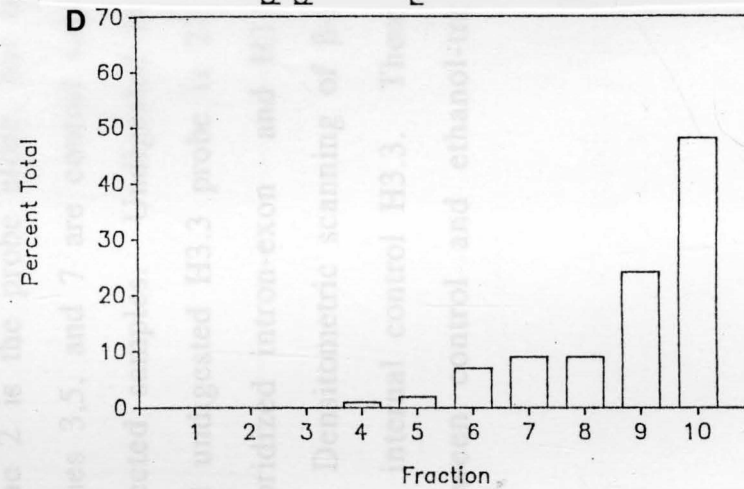
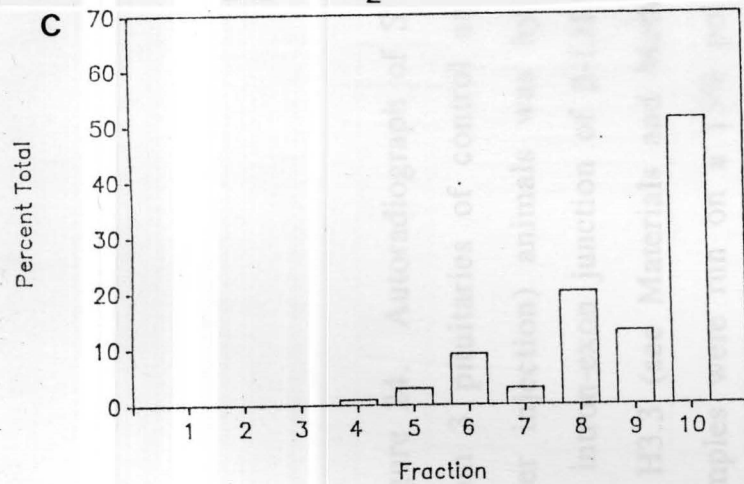
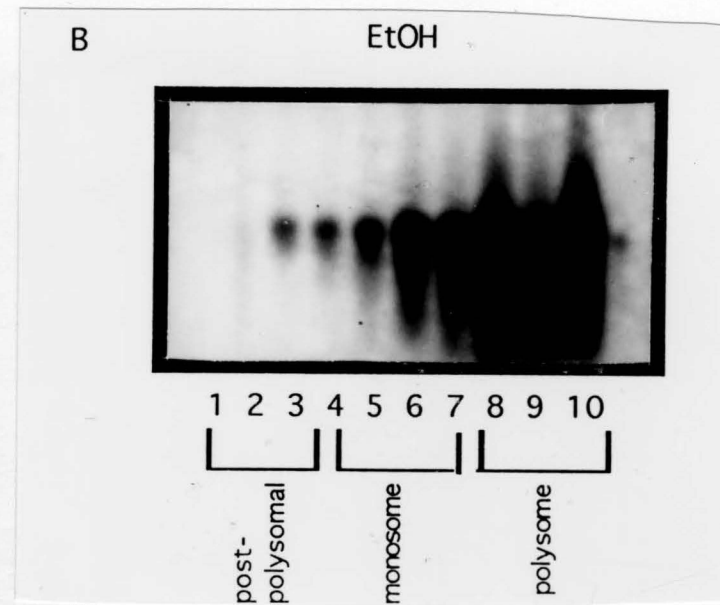
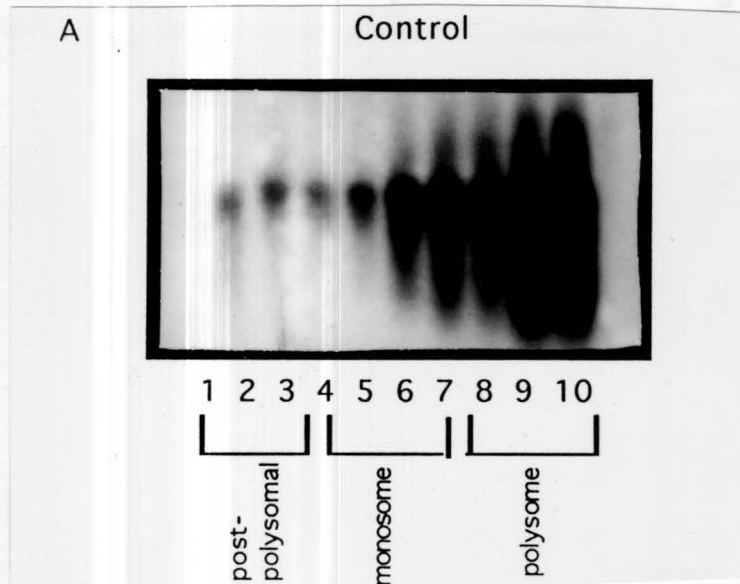
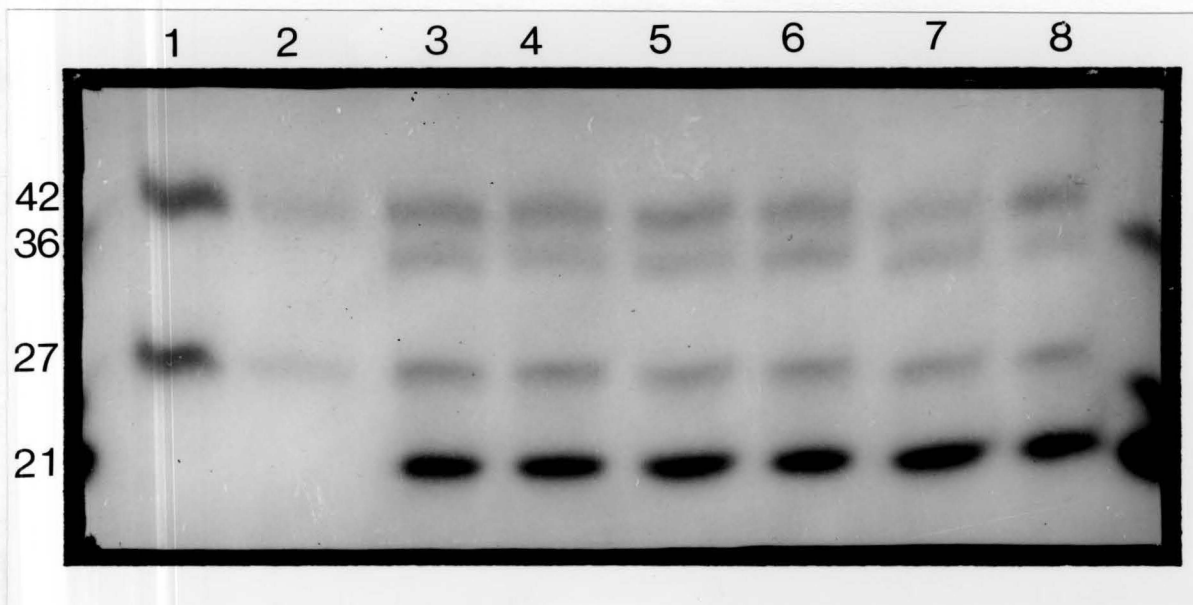
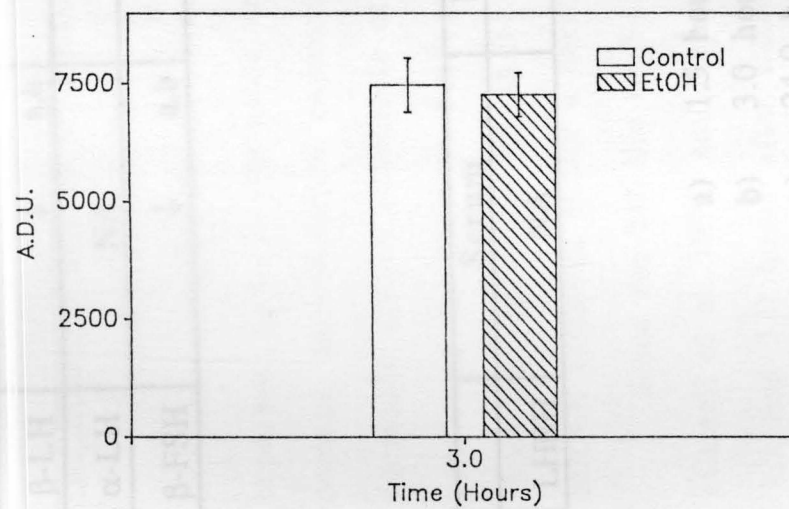


Figure 24. Autoradiograph of S1 Nuclease Protection Assay. (A) RNA from 3 pituitaries of control animals or ethanol-injected (3.0 hours after injection) animals was hybridized with oligo complementary to an intron-exon junction of β -LH mRNA and an oligo complementary to H3.3 (see Materials and Methods). After S1 nuclease digestions, samples were run on a 15% polyacrylamide gel and the gel was then exposed to film. Lane 1 is the probe alone without hybridization, Lane 2 is the probe alone, but treated the same as the samples. Lanes 3,5, and 7 are control samples. Lanes 4,6,8, are ethanol-injected samples. Undigested intron-exon probe is 42 nucleotides and undigested H3.3 probe is 27 nucleotides. Expected sizes for hybridized intron-exon and H3.3 probe are 36 and 21, respectively (B) Densitometric scanning of β -LH mRNA corrected for loading with the internal control H3.3. There was no significant difference found between control and ethanol-treated animals.

A**B**

Summary of Results

Table 1

	Serum	Pituitary	mRNA
β -LH	↓ a,b	↑ a	↓ a,b
α -LH	NA	NA	NC a,b,c
β -FSH	↓ a,b	NC a,b,c	NC a,b,c

Table 2

	Serum	Hypothalamic	mRNA
LHRH	NA	NC a,b,c	NC a,b,c

- a) 1.5 hours after ethanol injection
- b) 3.0 hours after ethanol injection
- c) 24.0 hours after ethanol injection

NC: no change

NA: not applicable

Table 1 and 2 represent a summary of the results of this research.

CHAPTER V

DISCUSSION

The Effect of Ethanol on Luteinizing Hormone

Previous studies have shown that acute ethanol exposure has an effect on pituitary endocrine function. Following an acute ethanol exposure, there is decreased serum LH, increased pituitary LH content, and decreased expression of the β -LH gene as shown by decreased mRNA (Emanuele et al. 1991). The present study confirmed the previous findings and are shown in Figures 3-6. The rapid and significant fall in β -LH mRNA suggests a decrease in β -LH mRNA synthesis and/or a decrease in β -LH mRNA stability.

Since the half-life of β -LH message is approximately 24 hours (Carroll et al. 1991) and the results show a decrease in β -LH mRNA 1.5 and 3.0 hours after ethanol exposure, the decreased steady-state level of β -LH mRNA may, at least partially, be due to an increase in mRNA degradation. Since the studies presented in my dissertation use an in vivo model, direct analysis of the half-life of β -LH mRNA in control and ethanol-treated samples is prohibitive. In order to evaluate half-life of β -LH mRNA, transcriptional inhibitors are applied and the amount of β -LH mRNA determined at various time points after treatment with the inhibitor. In in vivo studies, the

above is extremely difficult since it is impossible to determine whether the effects on the mRNA of interest is a result of the ethanol exposure or the result of the inhibitor on the animal itself.

There are several mechanisms by which the stability of β -LH could be decreased. It has been shown that the length of the poly(A) tail is of great importance in determining the stability of a hormone message (Nielsen and Shapiro 1990; Krane et al. 1991). The length of β -LH poly(A) tail was analyzed by Northern blot analysis (data not shown). There was no difference in the size of the transcript between control and ethanol samples. Even though the same blot reprobed with growth hormone, a transcript larger than β -LH, was able to detect a change in transcript size, the method employed may not be sensitive enough to detect a change in β -LH transcript size. Even if a change was detected in transcript size, this would only be indirect evidence that poly (A) tail modification was involved in the degradation of β -LH mRNA after acute ethanol exposure.

The transcriptional rate of β -LH mRNA may be altered by acute ethanol exposure. Nuclear Run-Off assays were performed to analyze this possibility. However, the levels of β -LH mRNA were too low to detect and a comparison of control and ethanol-treated β -LH mRNA transcriptional rate could not be made. In order to analyze the effects of ethanol on β -LH mRNA transcription, S1 nuclease protection assays were performed using a probe complementary to an intron-exon junction of β -LH mRNA. This probe was hybridized to newly transcribed mRNA which has not yet been processed

(heteronuclear RNA, hnRNA). This assay assumes equal splicing rates for control and ethanol-treated samples

A comparison of the amount of newly transcribed RNA for control and ethanol-treated samples using S1 nuclease protection found no significant difference. The samples used in this study were from animals injected with ethanol 3 hours previously. This is the same time point when β -LH mRNA was decreased 8-10 fold. This result does not completely exclude the possibility of β -LH mRNA transcriptional rate being altered by acute ethanol exposure. However, if the transcription of β -LH mRNA decreased, one would expect a decrease in the amount of hnRNA for β -LH in the ethanol-treated samples. This lack of suppression suggests ethanol actions are posttranscriptional. It should be noted that genomic DNA could hybridize to the intron-exon probe. However, the signal detected should not be altered significantly by such a small amount of genomic contamination.

Pituitary LH content was significantly increased in ethanol-treated animals compared to control rats, showing a greater than 200 percent increase 1.5 hours after injection. This increase occurred at a time when there was a decrease in serum LH and a decrease in β -LH mRNA. The increase in pituitary LH content without a subsequent increase in serum LH levels suggests impaired LH release or increase clearance of LH in the serum. The increase in pituitary LH content at the same time as a suppression of β -LH mRNA also suggests a decrease in secretion, as well as an alteration in the translation of the LH protein.

The impairment of LH release from the pituitary was addressed at the level of glycosylation of the LH protein. Since LH is a glycoprotein and glycosylation has been shown to affect transport and secretion of glycoproteins in cells (Tuma and Sorell 1988; Hoek and Rubin 1990; Ghosh et al. 1991), ethanol could affect the relative glycosylation of the LH protein. If the LH protein was modified by either an increase or decrease in glycosylation, a shift in the size of the LH protein would be expected when analyzed by Western blot analysis. The experiments completed have shown no shift in the LH protein size in the ethanol-treated samples. These results do not rule out the possibility that modifications of the LH protein are taking place. Other methods are needed to detect these changes.

Another possible mechanism of intracellular accumulation of LH protein is that the posttranslational trafficking of LH could be impaired by ethanol. Tuma and colleagues (1986) showed a markedly reduced secretion of glycoproteins in ethanol exposed livers. Further analysis of these tissues showed an accumulation of glycoprotein in the Golgi and endoplasmic reticulum (Tuma et al. 1986; Tuma and Sorell 1988). Subcellular fractionation of pituitaries followed by Western blot analysis, as seen in Figure 11, revealed no significant difference in the amount or size of LH protein in control and ethanol-treated samples. This result suggests the LH protein is processed properly through the Golgi. More conclusive results could be obtained by following the processing of radiolabeled protein through the endoplasmic reticulum and the Golgi apparatus.

The increase in pituitary LH content could also be due to effects of ethanol on translation of the LH protein. The translational efficiency can be analyzed using polysomal distribution analysis of β -LH mRNA. Polysomal distribution analysis enables one to detect actively translated messages by determining the amount of mRNA associated with the heavy polysome fractions in comparison to lighter polysome and monosome fractions. In this assay, sucrose gradient centrifugation is utilized to separate polysomes from monosomes. The RNA from these fractions is analyzed by Northern blot analysis. Control and ethanol samples were analyzed and their polysome profiles were compared. A reduced level of β -LH mRNA was found to be associated with heavy polysomes in the ethanol-treated samples. The α -subunit and β -FSH mRNA profile patterns were unaffected. Also the profile pattern for β -LH at the 24 hour time point was unaffected by ethanol. The shift of β -LH mRNA associating less with the heavier polysome fractions could expose the β -LH transcripts making them more susceptible to nucleases, and therefore degradation.

However, this shift in β -LH mRNA upon ethanol treatment is inconsistent with the increase in pituitary LH content, since a shift in the polysome profile would support a decrease in translational efficiency. Since a decrease in the translational efficiency was detected, this result supports the possibility that the increase in intrapituitary LH content is the result of posttranslational modification of the protein inhibiting its secretion from the gonadotrope cell.

In summary, there are three experimental findings which suggest that the decrease in steady-state mRNA levels are due to increase degradation of the β -LH transcript. First, the half-life of β -LH mRNA is approximately 24 hours. Second, no significant change in hnRNA for β -LH was found after ethanol exposure. Lastly, there is a shift in the polysome profile for β -LH after ethanol treatment. Furthermore, the polysome profile shift also implies that the effect of ethanol on the pituitary LH content takes place posttranslationally.

The Effect of Ethanol on Follicle-Stimulating Hormone

Ethanol caused significant falls in serum FSH 1.5 and 3.0 hours after injection, compared to controls. However, there was no significant difference in pituitary content of FSH between control and ethanol treated groups. The decrease in serum FSH could be the result of increased clearance of FSH in the serum or decreased secretion of FSH from the pituitary. Similar to LH, ethanol could be blocking FSH release through posttranslational modifications of the protein or altering intracellular trafficking of FSH in the pituitary cell. The target of ethanol's action being at the posttranslational level is supported by the polysome profiles obtained for β -FSH. The profiles, and therefore, the translational efficiency of β -FSH mRNA were unaffected by ethanol. Further analysis needs to be completed in these areas.

The steady-state β -FSH mRNA levels were not altered after ethanol treatment. This fact is in contrast to the dramatic decrease in β -LH mRNA at 1.5 and 3.0 hours after ethanol exposure. The

effect of ethanol on steady-state β -FSH mRNA appears to depend on the length of alcohol exposure. Studies have shown β -FSH mRNA to be altered by chronic alcohol exposure (Salonen et al. 1992). Ethanol's affect on the gonadotropin was not global in an acute ethanol exposure study. The above was also supported by the findings that the common α -subunit mRNA was unchanged after ethanol exposure. Since the gonadotropin messages have been shown to behave differently in response to the same stimuli and are also known to be regulated differently (Shupnik 1990; Weiss et al. 1993), it is not surprising to find FSH and LH affected differently by ethanol. The β -FSH message may be stabilized by some mechanism that is not used by β -LH. One possibility could be through the actions of activin. Activin, which has been shown to stabilize β -FSH mRNA, may play a role in protecting it from the degrading effects of ethanol (Carroll et al. 1991).

The Effect of Ethanol on LHRH mRNA and Hypothalamic Content

Ethanol was found not to have an effect on LHRH mRNA or LHRH hypothalamic content at any of the time points studied. I hypothesized that ethanol acted directly at the level of the hypothalamus decreasing LHRH mRNA levels. The results presented do not support this hypothesis. Based on these results and the information on the effects of ethanol on LHRH secretion from the hypothalamus in the portal blood (Emanuele et al. 1986; Emanuele et al. 1989), ethanol could be exerting its effects on LHRH secretion.

However, ethanol may also be affecting LHRH at the level of its receptor, altering its number and or its ability to bind LHRH.

Conclusion of Discussion

In conclusion, the main objectives of this dissertation were accomplished. Analysis of LH hnRNA levels after ethanol exposure suggests that the transcription of β -LH mRNA is not affected by ethanol. However, the translation of β -LH mRNA was shown to be affected by ethanol exposure, possibly resulting in an increase in degradation of β -LH mRNA. Whether or not the LH protein is modified as a result of exposure to ethanol could not be concluded from the studies completed. Finally, ethanol's effect on steady-state levels of mRNA seem to be at the level of the pituitary, since acute ethanol exposure did not affect LHRH mRNA levels. Even though the hypothalamic LHRH content was unaltered by ethanol exposure, the possibility exists that ethanol could be acting at a posttranslational level in the hypothalamus.

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